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Inheritance of fenoxaprop-P-ethyl resistance in a blackgrass (*Alopecurus myosuroides* Huds.) population

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Abstract A blackgrass population has developed resistance to fenoxaprop-P-ethyl following field selection with the herbicide for 6 consecutive years. Within this population, 95% of the individuals are also resistant to flupyrsulfuron. Both the inheritance(s) and the mechanism(s) of resistances were investigated by making crosses between the resistant and a susceptible biotype. The inheritance was followed through the F_1 and F_2 generations either by spraying the herbicide on seedlings at the three-leaf stage or using a seedling bioassay, based on coleoptile length. No maternal effects were evident in the fenoxaprop-P-ethyl responses of the F_1 plants, suggesting that the inheritance was nuclear. Some F_1 families treated with fenoxaprop-P-ethyl segregated in a 3:1 (resistant:susceptible) ratio, indicating that the resistance was conferred by two dominant and independent nuclear genes. This was confirmed by the 15:1 (R:S) ratio observed in the F_2 generation treated with fenoxaprop-P-ethyl. The use of selective inhibitors of herbicide detoxifying enzymes (aminobenzotriazole, pyperonylbutoxide, malathion and tridiphane) with the F_2 plants suggested that each of the two genes may govern two different mechanisms of fenoxaprop-P-ethyl resistance: the ACCase mutation previously postulated and an enhanced herbicide metabolism, mediated by cytochrome P 450 mono-oxygenases (P 450) susceptible to malathion. The P 450 activity may also confer resistance to flupyrsulfuron. This study clearly indicates that two distinct mechanisms of resistance may co-exist in the same plant.

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Introduction

Herbicide-resistant weeds were confirmed in the late 1960 s (Ryan 1970). Since then, herbicide resistance has been steadily increasing with approximately nine new cases per year (Heap 1999). Whereas, in 1982, in the first compilation of herbicide resistance, only 30 resistant species were reported (Bandeen et al. 1982), the 1999 international survey of herbicide-resistant weeds recorded more than 220 resistant biotypes in 45 countries around the world (Heap 1999).

Herbicide resistance can be defined as the ability of a biotype to survive a herbicide treatment to which the species is normally susceptible (LeBaron 1987; LeBaron and McFarland 1990; Maxwell and Mortimer 1994). Resistance is a heritable trait in the population and not a transient phenotypic response to an environmental condition which may allow plants to escape the herbicide effect (LeBaron and Gressel 1982). It is commonly accepted that herbicide-resistant weeds occurr naturally in populations at very low frequencies through recurrent mutation (Gressel and Segel 1978; Jasieniuk et al. 1995). These resistant plants will dominate a population only when they have a selective advantage in the presence of the herbicide. Therefore, repeated applications of a single herbicide, or herbicides with the same mode of action, provide the necessary selection pressure to shift weed populations toward high frequencies of resistant individuals. Herbicide resistance in weed species may be conferred by two major mechanisms: a modified herbicide target-site or an enhanced herbicide metabolism mediated by de-toxifying enzymes such as cytochrome P 450 mono-oxygenases (P 450) or glutathione transferases (GST) (Boustalis and Powles 1995; Devine 1997). Except for target-site triazine resistance, which is maternally inherited (Machado 1982), resistance to other herbicides most-commonly result from an alteration in a

single nuclear gene (Jasieniuk et al. 1995). One notable exception is chlortoluron resistance in blackgrass (*Alopecurus myosuroides*), which is controlled by at least two additive genes (Chauvel 1991). Moreover, in most instances where resistance is encoded by a nuclear gene, it is expressed as either a dominant or a partially dominant trait. This is true for sulfonylurea herbicide-resistant *Lactuca* spp. (Mallory-Smith et al. 1990), as well as for acetyl CoA carboxylase-resistant wild oat (*Avena fatua* L.) (Murray et al. 1995), diclofop-methyl-resistant italian ryegrass (*Lolium multiflorum*) (Betts et al. 1992) or acetolactate synthase-resistant common cocklebur (*Xanthium strumarium*) (Ohmes and Kendig 1999). An exception is dinitroaniline resistance in green foxtail (*Setaria viridis*) (Jasieniuk et al. 1994) and in goosegrass (*Eleusine indica*) (Zeng and Baird 1997), which is inherited as a recessive character.

Since 1960, blackgrass has been a common annual grass-weed in autumn-sown crop rotations of Atlantic European countries (Melander 1995; Chauvel et al. 2000), and it is widely known that the infestation level of blackgrass tends to increase when the proportion of winter cereals in the crop rotation is increased (Hurle 1993). In France, blackgrass populations have been reported to be particularly hard to control in winter cereal crops with fenoxaprop-P-ethyl, an inhibitor of the acetyl CoA carboxylase (ACCase), a key enzyme involved in fatty acid biosynthesis (Secor and Cséke 1988). Since 1993, the existence of more than 212 fenoxaprop-P-ethyl-resistant populations has been reported, mainly in the north of France (Gasquez 1998). As blackgrass is highly allogamous, most of the resistant individuals are heterozygous for the resistance trait and populations are frequently made up of a mixture of susceptible and resistant plants (Chauvel and Gasquez 1994; Gasquez 1996; Letouzé et al. 1997). In 1996, a blackgrass population, highly resistant to fenoxaprop-P-ethyl, was reported in Burgundy (France). This population had been selected by an intensive and exclusive use of fenoxaprop-P-ethyl for 6 consecutive years and the individuals are resistant to ten-fold the recommended herbicide dose, probably due to an ACCase mutation (Letouzé and Gasquez 1999, 2000). Moreover, the majority of these fenoxaprop-P-ethyl-resistant plants are also resistant to flupyrsulfuron, an acetolactate synthase (ALS) inhibitor, when they have never been exposed to any other sulfonyurea herbicides (Letouzé and Gasquez 1998). Although the resistance mechanism to flupyrsulfuron is still unknown, it is likely that this resistance has been selected by the intensive use of fenoxaprop-P-ethyl.

The objectives of the present study were, firstly, to determine the inheritance of fenoxaprop-P-ethyl resistance in this blackgrass population and, secondly, to investigate the mechanisms of resistance involved in the population. To reach this goal, reciprocal crosses were made between the resistant and susceptible plants, and resistance segregation in the F_1 and F_2 generations was analysed. The resistance mechanism(s) to fenoxaprop-P-ethyl and flupyrsulfuron were then investigated in the F_2 plants using a seedling bioassay with selective inhibitors of the herbicide de-toxifying enzymes P 450 mono-oxygenase (P 450) and glutathione transferases (GST). Understanding the genetics of herbicide resistance, as well as the mechanisms of resistance in weeds, will aid in predicting and possibly controlling the spread of the herbicide-resistant plants.

Materials and methods

Plant material

General procedures

Seeds were pre-germinated in 15 cm-diameter glass Petri-dishes lined with small glass tubes supporting one sheet of blotting paper (Germaflor No. 55, 160 g/m^{-2}, Müller). To stimulate seed germination, Petri-dishes were filled with 30 ml of 2 g KNO_3 l^{-1} and placed in a controlled environment room (12-h, 20°C light/12-h, 15°C dark). Each seedling was transplanted at the one-leaf stage to a pot (10 cm×10 cm×10 cm) containing potting soil (coarse sand and peat 1:3, v/v) and kept outdoors during the winter growing season to induce flowering. Before flowering, plants were transferred to the greenhouse (14-h, 22°C light/10-h, 12°C dark) to make the controlled crosses.

Parental populations

Susceptible (S) and fenoxaprop-P-ethyl-resistant (R) parents, both collected in Burgundy (France), were used to conduct this inheritance study. The S parents came from a 100% susceptible population whereas the R parents were screened from a 100% resistant population selected by the intensive and exclusive use of fenoxaprop-P-ethyl for 6 years. A large majority (95%) of these fenoxaprop-P-ethyl-resistant parents were also resistant to flupyrsulfuron (Letouzé and Gasquez 1998; Letouzé et al. 1999). A total of 150 seeds from both the S and R populations were germinated as described above. The fenoxaprop-P-ethyl-resistant parents involved in the crosses were selected by spraying 690 g a.i. ha^{-1} of fenoxaprop-P-ethyl (as "Puma S" – 69 g a.i. L^{-1}, AgrEvo) with a laboratory sprayer at the three-leaf stage as described by Letouzé et al. (1997). This high herbicide dose was used to screen the highly resistant parents. All the selected R plants survived this herbicide concentration (ten-fold the normal recommended dose) (Letouzé and Gasquez 2000). Among these fenoxaprop-P-ethyl resistant plants, 95% were also resistant to two-fold the recommended dose of flupyrsulfuon (159 g a.i. ha^{-1} as "Lexus XPE", 33%; Dupont). The S and R parents were also selected according to contrasting homozygous isozyme genotypes before they were used in crosses (see below).

Development of the F_1 generation

To produce F_1 seeds, parental plants were germinated and grown as described in the "general procedures" section and 15 reciprocal crosses were made by hand in the greenhouse between the S and R selected parents. Crosses were carried out by enclosing a spike from each S and R parent within a bag 1 day before anthesis. At the end of the flowering time, the bag was taken off to ensure good seed maturation. The F_1 seeds from each parental spike involved in the cross were harvested at maturity as individual seed lots.

Development of the F_2 generation

The F_2 generation was produced by making crosses between F_1 plants from two different families consisting entirely of fenoxa-

prop-P-ethyl-resistant individuals. Crosses were made between F_1 plants from the same family or between individuals from each family. Crosses and harvest of the F_2 seeds were conducted as described above.

F_1 hybrid identification

Although blackgrass is a highly allogamous species, a low level of self- pollinisation may occur (Chauvel and Gasquez 1994). Thus, to differentiate true F_1 hybrids from "selfed" ones, the S and R parents were selected based on contrasting homozygous isozyme genotypes. The superoxide dismutase (SOD) locus is polymorphic in blackgrass and is represented by two alleles ("lower-band" allele and "upper-band" allele) (Chauvel 1991). Parents selected for each cross had different alleles at the SOD locus. As the SOD enzyme is dimeric (Fridovich 1975), the heterozygous genotype of the true F_1 hybrids will be three-banded. The mixture of true hybrids and "selfed" seeds produced from each of the reciprocal crosses were germinated and grown as described in the "General procedures" section and 420 F_1 seedlings were screened for hybridity at the three-leaf stage by conducting isozyme electrophoresis (Gasquez and Compoint 1976) and SOD staining (Chauvel 1991).

Resistance screening procedures in the F_1 and F_2 generations

Spraying of herbicide

At the three-leaf stage, the true F_1 seedlings, identified using the SOD marker, were sprayed with 138 g a.i. ha^{-1} of fenoxaprop-P-ethyl (two-fold the lethal dose) with a laboratory sprayer as described by Letouzé et al. (1997). This herbicide dose, lower than the one used to screen the R parents, allowed the selection of both the slightly and highly R F_1 plants (Letouzé and Gasquez 1999). The S and R phenotypes of the F_1 plants were defined 4 weeks after the herbicide treatment: the individuals that were killed by the herbicide treatment were classified as susceptible (S) and those that survived the herbicide treatment were classified as resistant (R). The R F_1 hybrids which originated from the 100% R F_1 families were then used for making crosses to produce the F_2 generation.

Seedling bioassay

A seedling bioassay, based on the coleoptile length after a 6-days-growth in 6 $mg \cdot l^{-1}$ of fenoxaprop-P acid (AE F088406 00 IC94 0001, AgrEvo, Germany) was used to screen resistance in the F_2 generation (Letouzé and Gasquez 1999). According to this herbicide resistance screening test, the coleoptile length of S and R seedlings are shorter and longer than 10 mm, respectively. This seedling test also allows the distinction between two kinds of fenoxaprop-P-ethyl-resistant plants: the "highly" resistant (Rh) with a coleoptile longer than 20 mm and the "moderately" resistant (Rm) with a coleoptile between 10 and 20 mm (Letouzé and Gasquez 1999). The S and R phenotype of 450 F_2 plants originating from crosses between F_1 plants coming from the 100% R F_1 families was defined using this seedling test.

Mechanims of resistance

Fenoxaprop-P-ethyl

To investigate the mechanism(s) of resistance involved in the R population, the seedling test was conducted with the F_2 generation using four selective inhibitors of P 450 mono-oxygenases (P 450) and gluthatione transferases (GST). The inhibitors used were: 1-aminobenzotriazole (ABT) (Sigma, France), piperonyl-butoxide (PBO) (Loveland Industries, USA) and malathion (Cluzeau, France) as P 450 inhibitors (Christopher et al. 1994; Gaillardon et al. 1985; Singh et al. 1998), and tridiphane (AGER 280959 Dowelanco, USA) as a GST inhibitor (Lamoureux et al. 1986). ABT (10 $mg \cdot l^{-1}$), PBO (20 $mg \cdot l^{-1}$), malathion (20 $mg \cdot l^{-1}$) and tridiphane (0.625 $mg \cdot l^{-1}$) were used in combination with 6 $mg \cdot l^{-1}$ of fenoxaprop-P acid. For each inhibitor, the concentration selected was the highest among a range of doses that had no phytotoxic effect on its own. (Letouzé 1999). Thus, when the growth of a resistant coleoptile is affected, it only results from the inhibition of the target de-toxification enzyme used in combination with the herbicide. Fifty F_2 seeds from the same crosses used in the seedling test were grown for 6 days in the presence of one of the four combinations "fenoxaprop-P acid+inhibitor." The inhibitor effect was then assessed by measuring the coleoptile length.

Flupyrsulfuron

A similar experiment was conducted with the F_2 generation to characterize the cross-resistance to flupyrsulfuron of the R population. These F_2 seedlings came from the same F_1 crosses used to investigate the mechanism of fenoxaprop-P-ethyl resistance. A dose of 320 $mg \cdot l^{-1}$ of flupyrsulfuron (as "Lexus XPE", 33%; Dupont) was used with each inhibitor concentration. This flupyrsulfuron concentration has been previously selected to allow a reliable discrimination between susceptible (S) and resistant (R) seedlings: the R coleoptiles are longer than 10 mm whereas the S coleoptiles never exceed 10 mm (Letouzé 1999; Letouzé et al. 1999).

Statistical analysis

For each generation, the observed segregation ratios of susceptible and resistant phenotypes were tested for goodness of fit to expected Mendelian ratios using a chi-square test (Scherrer 1984). Homogeneity chi-square tests were performed to determine if the data could be pooled within crosses and between reciprocal crosses (Scherrer 1984).

Results

Inheritance of resistance to fenoxaprop-P-ethyl

F_1 results

The phenotype of the F_1 seedlings generated from 15 reciprocal crosses between susceptible (S) and resistant (R) plants was determined by spraying with 138 $g.ha^{-1}$ of fenoxaprop-P-ethyl. The two parental phenotypes were observed: a R phenotype with slight or no injury and a S phenotype that was killed by the herbicide treatment. No significant differences were found in the fenoxaprop-P response of F_1 plants generated from reciprocal crosses (data not shown). Thus, data from each reciprocal cross have been pooled. Based on data from the spray test, three different types of F_1 families were observed (A, B and C) and a homogeneity chi-square test indicated that there was no significant difference in the fenoxaprop-P response of families within each group (Table 1). Group A, made up of three F_1 families (173 F_1 plants), segregated in 89 R and 84 S individuals, group B, made up of ten F_1 families (247 plants), segregated in 196 R and 51 S plants, whereas two F_2 families in group C (70 F_1 plants) consisted entirely of plants resistant to 138 $g.ha^{-1}$ of fenoxaprop-P-ethyl (Table 1). The uniform expression of a R parental phenotype in the F_1 families from group C led to the conclusion that fenoxaprop-P-

Table 1 Segregation for fenoxaprop-P-ethyl resistance in the three groups of F_1 families generated from crosses between resistant (R) and susceptible (S) parents

[a] Expected Mendelian segregation ratio between S and R phenotypes for each F_1 family group

F_1 family groups	No. of F_1 families	Number of seedlings			R:S[a]		χ^2	Probability
		R	S	Total				
A	3	89	84	173	1:1		0.15	0.70
B	10	196	51	247	3:1		2.42	0.12
C	2	70	0	70	1:0		0	1
Test of homogeneity among F_1 families within each group						A	0.04	0.98
						B	4.93	0.84
						C	0	1

Table 2 Segregation for fenoxaprop-P resistance in the F_2 generation generated from crosses between resistant F_1 plants from group C. S: susceptible; R: resistant; Rh: "highly" resistant; Rm: "moderately" resistant

[a] Based on coleoptile length (CL, mm) in the seedling test

F_2 crosses	Number of seedlings			Expected ratio	χ^2	Probability
	R		S			
	Rh CL>20[a]	Rm 10<CL<20[a]	CL<10[a]			
1	34	12	4	12:3:1	1.31	0.52
2	38	10	2	12:3:1	0.45	0.80
3	33	12	5	12:3:1	2.40	0.30
4	39	9	2	12:3:1	0.48	0.79
5	37	10	3	12:3:1	0.05	0.97
3	32	13	5	12:3:1	3.33	0.19
7	36	10	4	12:3:1	0.35	0.84
8	37	11	2	12:3:1	0.69	0.71
9	35	12	3	12:3:1	0.91	0.64
Observed	321	99	30	12:3:1	3.47	0.18
Test of homogeneity among F_2 crosses					6.61	0.98

ethyl resistance is dominant. The absence of 100% S F_1 families supports this conclusion. Furthermore, results from group C suggested that the two R parents that generated these 100% R F_1 families were homozygous for the resistance trait. A chi-square test indicated that F_1 families from group A segregated in a 1:1 resistant: susceptible (R:S) ratio (P=0.7), indicating that the resistance of the R parents involved in these crosses is governed by a single nuclear dominant gene (Table 1). The F_1 families from group B segregated in a 3:1 (R:S) pattern (P=0.12, Table 1). Therefore, the R parents which have generated these F_1 families appear to have, at least, two nuclear dominant genes endowing resistance. Moreover, the presence of both R and S parental phenotypes in the F_1 families from group A and B suggested that the R parents involved in these crosses were heterozygous for the fenoxaprop-P-ethyl resistance trait. Therefore, the F_1 experiment suggested that some R parents are resistant to fenoxaprop-P-ethyl due to one resistant, gene whereas others possess at least two resistant genes.

F_1 results

To further confirm the hypothesis that fenoxaprop-P-ethyl resistance is governed by at least two nuclear dominant genes for the majority of the R parents, we followed the inheritance of resistance through the F_2 generation. Segregation studies were conducted using F_2 progenies originating from crosses between F_1 plants that had survived 138 g.ha^{-1} of fenoxaprop-P-ethyl and where the R parent was presumed to be homozygous for the resistance trait (F_1 plants from group C). The resistance segregation was determined for nine F_2 crosses using the seedling test. For all the F_2 crosses tested, when seedlings were exposed to 6 mg· l^{-1} of fenoxaprop-P-ethyl for 6 days, three distinct responses were observed based on the coleoptile length: (1) seedlings with a coleoptile smaller than 10 mm, (2) seedlings with a coleoptile between 10 and 20 mm long, and (3) seedlings with a coleoptile longer than 20 mm (Table 2). These F_2 seedlings were classified as phenotypically susceptible (S), "moderately" resistant (Rm) and "highly" resistant (Rh), respectively. For fenoxaprop-P-ethyl, both S and Rh phenotypes were parental (Table 3), whereas the Rm phenotype was intermediate. All the F_2 crosses were made of S, Rm and Rh seedlings and a homogeneity chi-square test indicated that there were no significant differences between the fenoxaprop-P response of the nine F_2 crosses (Table 2). Thus, data from each family were pooled (Table 2). If resistance was due to two independent dominant genes (gene 1 and gene 2) with two alleles ("R" for the resistant allele and "r" for the susceptible allele), and provided that either of the two resistant alleles (R1 or R2) respectively confers a high and a moderate level of resistance, the F_2 crosses tested should segregate in a 12:3:1 (Rh:Rm:S) ratio as shown by the theoretical model in Fig. 1A. Among the F_2 plants from the seedling test (450 plants), 321 were "highly" resistant, 99 were "moderately" resistant and 30 were susceptible (Table 2).

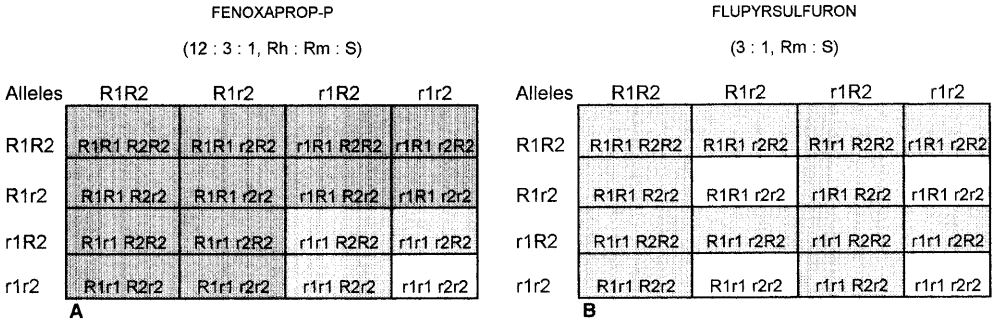

Fig 1 Segregation pattern and expected phenotypes for a two gene model for F_2 seedlings exposed for 6 days to 6 mg·l^{-1} of fenoxaprop or 320 mg·l^{-1} flupyrsulfuron R1=resistant allele for gene 1 conferring a high level of resistance to fenoxaprop-P, r1=susceptible allele for gene 1conferring susceptibility to both herbicides, R2=resistant allele for gene 2 conferring a moderate level of resistance to both fenoxaprop-P and flupyrsulfuron, r2=susceptible allele for gene 2 conferring susceptibility to both herbicides.Phenotype: ■ "highly" resistant (*Rh*) ■ "moderately" resistant (*Rm*) □ susceptible (*S*)

Table 3 Coleoptile lengths (CL, mm) after 6 days growth without herbicide, on 6 mg·l^{-1} of fenoxaprop-P or 320 mg·l^{-1} of flupyrsulfuron for the susceptible (S) and resistant (R) populations used as parents in the inheritance study

Herbicide	Populations	Number of seedlings		
		CL<10	10<CL<20	CL>20
None	R	0	0	100
	S	0	0	100
Fenoxaprop-P	R	0	0	100
	S	100	0	0
Flupyrsulfuron	R	5	95	0
	S	100	0	0

These observed ratios were not significantly different from the predicted 12:3:1 (Rh:Rm:S) ratio (P=0.18; Table 2). Thus, these results confirm the hypothesis drawn from the F_1 experiment that fenoxaprop-P-ethyl resistance of most of the R parents is controlled by two independent nuclear dominant genes, and indicated that each of the two dominant alleles, R1 and R2, respectively confers a high and a moderate level of resistance to fenoxaprop-P-ethyl.

Mechanism of resistance

Resistance to fenoxaprop-P-ethyl

The distinction between the two resistant phenotypes (Rh and Rm) in the studied F_2 generation suggested that each of the two genes conferring fenoxaprop-P-ethyl resistance might govern a distinct mechanism of resistance. Therefore, to test the hypothesis that several mechanisms coexists in the R population, the seedling test was conducted with seven of the nine F_2 crosses using selective inhibitors of P 450 mono-oxygenases (P 450) and glutathione transferases (GST) (Table 4).

Table 4 Segregation for fenoxaprop-P resistance in the F_2 generation originating from crosses between resistant F_1 plants from group C after 6 days of growth with the seedling test in the herbicide and a detoxifying enzyme inhibitor. S:susceptible; Rh:"highly" resistant; Rm:"moderately" resistant; ABT: aminobenzotriazole; PBO: pyperonyl butoxide

Inhibitor	Phenotypes	F_2 crosses							Total	Expected ratio	χ^2	Probability
		1	2	3	4	5	6	7				
		Number of individuals										
ABT	Rh	36	39	37	37	34	38	38	259	12		
	Rm	11	8	11	10	13	10	9	72	3	1.02	0.6
	S	3	3	2	3	3	2	3	19	1		
	Test of homogeneity among F_2 crosses										4.18	0.98
PBO	Rh	33	40	36	38	33	35	34	249	12		
	Rm	13	8	11	10	12	11	12	77	3	2.69	0.26
	S	4	2	3	2	5	4	4	24	1		
	Test of homogeneity among F_2 crosses										5.23	0.95
Tridiphane	Rh	34	38	38	36	34	36	35	251	12		
	Rm	12	8	10	11	13	10	12	76	3	2.77	0.25
	S	4	4	5	3	3	4	3	26	1		
	Test of homogeneity among F_2 crosses										4.18	0.98
Malathion	Rh	34	39	37	37	35	37	36	255	3		
	Rm	0	0	0	0	0	0	0	0	0	2.10	0.35
	S	16	11	13	13	15	13	14	95	1		
	Test of homogeneity among F_2 crosses										4.60	0.97

For each inhibitor, there was no significant difference in the response of the seven F_2 crosses (P_{ABT}=0.98; P_{PBO}=0.95; $P_{tridiphane}$=0.98; $P_{malathion}$=0.97), so data were pooled across crosses (Table 4). When the F_2 seedlings were grown in the presence of fenoxaprop-P with either aminobenzotriazole (ABT), piperonylbutoxide (PBO) or tridiphane, the three Rh, Rm and S phenotypes could be observed and resistance segregated in a 12: 3: 1 (Rh: Rm: S) ratio (Table 4), similar to the one observed when fenoxaprop-P was used alone (Table 3). This result suggested that none of the de-toxifying enzymes (P 450 or GST) inhibited by ABT, PBO or tridiphane are involved in the resistance of these F_2 seedlings. When malathion was used, the Rm phenotype was no longer observed whereas the two parental phenotypes (S and Rh) were still detected (Table 4). Among the 350 F_2 seedlings grown with fenoxaprop-P and malathion, 255 were "highly" resistant and 95 were susceptible to fenoxaprop-P-ethyl (Table 4). A chi-square test indicated that these two parental phenotypes segregated in a 3:1 (Rh:S) ratio within the F_2 generation (Table 4), suggesting that all the Rm F_2 seedlings became susceptible in the presence of malathion, whereas the Rh ones were not inhibited by malathion and fenoxaprop-P. This result suggested that P450 inhibited by malathion is responsible for the fenoxaprop-P-ethyl resistance of the Rm F_2 seedlings and that another mechanism confers resistance to the Rh seedlings. According to the theoretical model shown in Fig. 1A, 3/16 of the F_2 seedlings possess the R2 allele when the R1 allele is absent. The Rm F_2 seedlings are moderately resistant to fenoxaprop-P due to P 450 activity, whatever their genotype is, either homozygous (*r1r1 R2R2*), or heterozygous (*r1r1 R2r2*). Furthermore, Fig. 1A indicates that 12/16 of the F_2 seedlings possess at least one copy of the R1 allele whether the R2 allele is, or is not, present. Thus, these R F_2 seedlings might be those that still displayed the "highly" resistant parental phenotype in the presence of malathion and may, at least, possess the ACCase mutation, previously postulated (Letouzé and Gasquez 2000). Therefore, this F_2 experiment confirmed that the two dominant resistant genes (R1 and R2) governed two distinct mechanisms of fenoxaprop-P-ethyl resistance that could be: an ACCase mutation, conferring a high level of resistance as suggested earlier (Letouzé and Gasquez 2000), and enhanced herbicide metabolism mediated by P 450 susceptible to malathion conferring a lower level of resistance.

Resistance to flupyrsulfuron

When the fenoxaprop-P-ethyl resistant parents used for the inheritance study were exposed for 6 days to 320 mg·l^{-1} of flupyrsulfuron, two types of phenotype were observed: (1) seedlings with a coleoptile smaller than 10 mm, as in the S population, and (2) seedlings with coleoptiles between 10 and 20 mm long, as in the majority of the R parents (Table 3). These two parental phenotypes have been classified as "susceptible" (S) and "moderately" resistant (Rm). As revealed by the spray test (data not shown), the seedling test confirmed that 95% of the fenoxaprop-P-ethyl-resistant parents are also resistant to flupyrsulfuron, whereas the other 5% are only resistant to fenoxaprop-P-ethyl (Table 3).

To understand flupyrsulfuron cross-resistance, the seedling test was conducted with the four inhibitors using 50 F_2 seeds coming from the same F_2 crosses used for the fenoxaprop-P-ethyl experiment (Table 5). Data for each inhibitor were pooled since there were no significant differences in the response of the seven F_2 cross-

Table 5 Segregation for flupyrsulfuron resistance in the F_2 generation originating from crosses between resistant F_1 plants from group C after 6 days of growth with the seedling test in the herbicide and a de-toxifying enzymes inhibitor. S: susceptible; Rh:"highly" resistant; Rm:"moderately" resistant; ABT: aminobenzotriazole; PBO: pyperonyl butoxide

Inhibitor	Phenotypes	F_2 crosses							Total	Expected ratio	χ^2	Probability
		1	2	3	4	5	6	7				
		Number of individuals										
None	Rm	40	36	42	39	32	33	34	256	3	0.65	0.42
	S	10	14	8	11	18	17	16	94	1		
		Test of homogeneity among F_2 crosses									1.64	0.95
ABT	Rm	42	37	36	38	37	39	40	269	3	0.25	0.62
	S	8	13	14	12	13	11	10	81	1		
		Test of homogeneity among F_2 crosses									1.33	0.97
PBO	Rm	33	40	36	37	41	35	37	259	3	0.57	0.45
	S	17	10	14	13	9	15	13	91	1		
		Test of homogeneity among F_2 crosses									1.88	0.93
Tridiphane	Rm	33	38	41	35	36	42	32	257	3	0.99	0.32
	S	17	12	9	15	14	8	18	93	1		
		Test of homogeneity among F_2 crosses									1.64	0.95
Malathion	Rm	0	0	0	0	0	0	0	0	0	0	1
	S	50	50	50	50	50	50	50	350	1		

es (Table 5). When F_2 seedlings were exposed to flupyrsulfuron with either ABT, PBO or tridiphane a 3:1 (Rm:S) ratio was observed (Table 5). This ratio was similar to the one observed when the F_2 seedlings were exposed to flupyrsulfuron alone (Table 5). This result indicated that none of the de-toxifying enzymes inhibited by these products are responsible for the flupyrsulfuron resistance of the R F_2 seedlings. When exposed to flupyrsulfuron and malathion, all the F_2 seedlings tested for resistance became susceptible (Table 5). This suggests that P 450 inhibited by malathion is involved in flupyrsulfuron resistance of the R F_2 seedlings. According to the theoretical model shown in Fig. 1, 3/4 of the F_2 seedlings possess at least one copy of the R2 allele. Thus, these seedlings must be those that are moderately resistant to flupyrsulfuron due to P 450. Furthermore, Fig. 1 also indicates that 1/4 of the F_2 seedlings do not possess the R2 allele. Thus, these seedlings might be those that are susceptible to flupyrsulfruon, whether the R1 allele presumed to be responsible for the ACCase mutation is present or not. As all the R parents used for this study are supposed to have a ACCase mutation (Letouzé and Gasquez 2000), the F_2 experiment suggested that the R parents that are highly resistant to fenoxaprop-P-ethyl but do not show cross-resistance to flupyrsulfuron (Table 4) should only possess the R1 allele responsible for the ACCase mutation. The other 95% of individuals, which are resistant to both fenoxaprop-P-ethyl and flupyrsufuron (Table 3), may be resistant to flupyrsulfuron due to the same P 450 that confers resistance to fenoxaprop-P-ethyl.

Discussion

This study was conducted to determine the inheritance of fenoxaprop-P-ethyl and distinguish the mechanism(s) of resistance involved in a blackgrass population selected following an intensive use of the herbicide. The absence of reciprocal differences in F_1 plants demonstrated that the genetic control of fenoxaprop-P-ethyl resistance is nuclear and not cytoplasmic, while the segregation of resistance in the F_1 and F_2 generations suggested that at least two dominant and independant genes are responsible for resistance in the majority of the resistant (R) parents (Tables 1 and 2). In addition, the non-parental fenoxaprop-P phenotype called moderately resistant (Rm) suggested that these two genes govern a distinct mechanism of resistance, each one conferring a different level of fenoxaprop-P-ethyl resistance (high and moderate).

Up to now, the only mechanism of resistance postulated in the fenoxaprop-P-ethyl-resistant blackgrass population used in this study was an Accase mutation (Letouzé and Gasquez 2000). The segregation of genes in the F_2 generation and the use of selective inhibitors of de-toxifying enzymes revealed that another mechanism of resistance coexists in the R population. Indeed, the seedling test conducted with F_2 plants exposed to fenoxaprop-P-ethyl and malathion indicated that the majority of the R parents are also resistant to this ACCase inhibitor, probably due to the enhanced herbicide metabolism mediated by P 450 mono-oxygenases (P 450) susceptible to malathion (Table 4). Therefore, this study suggested that each of the two resistant, nuclear, dominant genes governs a distinct mechanism of resistance. Our results are also strong evidence that two mechanism of resistance may coexist in the same plant. Indeed, the herbicide treatment of a large polymorphic population, like the blackgrass population, may result in the survival of individuals that possess one or more (different) mechanisms of resistance. Therefore, with out-crossing species like blackgrass, there is gene flow among the survivors, resulting in the exchange of different resistance genes and their accumulation in the next generation.

To-date, the only reported mechanism of enhanced fenoxaprop-P-ethyl metabolism was mediated by glutathione transferases (GST) within the "Peldon" blackgrass population from England (Cummins et al. 1997). Thus, this is the first time that a mechanism of resistance to fenoxaprop-P-ethyl related to P 450 activity is suggested within blackgrass.

Moreover, the seedling test conducted with F_2 plants exposed to flupyrsulfuron in association with the four inhibitors revealed that P 450 inhibited by malathion could also be responsible for the resistance to flupyrsulfuron in 95% of the fenoxaprop-P-ethyl-resistant parents, whereas they have never been exposed to this herbicide. Thus, our results suggested that the P 450 enzyme conferring resistance to flupyrsulfuron may have been selected by the intensive use of fenoxaprop-P-ethyl for 6 consecutive years. The activity of P 450 inhibited by malathion has already been reported to be responsible for the resistance to a sulfonylurea herbicide within rye grass (*Lolium rigidum*) (Christopher et al. 1994). Indeed, this biochemical study revealed that malathion increased chlorsulfuron toxicity by inhibiting the herbicide metabolism mediated by P 450. As a single P 450 conferred resistance to both fenoxaprop-P-ethyl and flupyrsulfuron, our work suggests that such de-toxifying enzymes are not selective of one herbicide or one herbicide family.

Many studies of inheritance in other species having a ACCase mutation, such as Italian ryegrass (*Lolium multiflorum*) (Betts et al. 1992), wild oat (*Avena fatua*) (Murray et al. 1995) and foxtail millet (*Setaria italica*) (Wang et al. 1996), also indicated that resistance was due to monogenic dominant inheritance. On the other hand, very few studies were conducted to examine the inheritance of enhanced herbicide metabolism. The only investigation carried out was with a blackgrass population resistant to chlortoluron (Chauvel 1991) and it was suggested that resistance was controlled by at least two additive genes. Thus, to confirm that the enhanced fenoxaprop-P-ethyl metabolism mediated by P 450 is controlled by a single gene, crosses between the S parents and the F_2 seedlings resistant to flupyrsulfuron should be made.

As we wanted to use all the fenoxaprop-P-ethyl-resistant phenotypes to conduct the inheritance studies in the

F_1 and F_2 generations, the fenoxaprop-P-ethyl-resistant parents have only been sprayed with two-fold of the herbicide recommended dose rather than a range of doses. Thus, dominance or semi-dominance of the two resistant genes has not been investigated. Nevertheless, the spray test indicates that all the R parents (homozygous or heterozygous) are resistant to ten-fold above the recommended dose of fenoxaprop-P-ethyl, and that the majority of them are also resistant to two-fold the recommended dose of flupyrsulfuron. Thus, one allele responsible for the P 450 activity (designed as R2) or one allele encoded for the ACCase mutation (designed as R1) seems to confer resistance to higher doses than used by farmers in the field. Therefore, under normal field-selection conditions, resistance in a population like the one used in this study would be expressed as a fully dominant trait. Whatever the genotype, either homozygous or heterozygous for one gene or two, the plants will survive under field conditions and will transfer the resistance trait, via pollen transfer, to the next generation. The dominant expression of the resistance genes at the recommended herbicide dose will accelerate the spread of resistance within a population compared to the spread of a recessive resistance gene. In addition, because blackgrass is highly allogamous, the spread of resistance is strongly increased compared to what would be observed for a self-pollinated species. Based on the mode of inheritance for the resistance trait, one would expect that resistance would evolve rapidly in the field given successive applications of fenoxaprop-P-ethyl or flupyrsulfuron. This finding supports the experiment carried out by Chauvel et al. (1992) with a blackgrass population under greenhouse conditions. In these conditions, only 4 years of intensive use of fenoxaprop-P-ethyl were sufficient to select a 100% fenoxaprop-P-ethyl-resistant population. Finally, this work demonstrates that several mechanisms of resistance, selected by the same herbicide, may coexist in a population and even in the same plant. This phenomenon, already observed in a rye grass populations (Preston et al. 1996), complicates the chemical management of these resistant weed populations. For such a population that accumulates several mechanisms of resistance, the use of mixtures of herbicides and cultural methods appears be the most-effective resistance management strategy.

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