Enhanced activity of several herbicide-degrading enzymes: a suggested mechanism responsible for multiple resistance in blackgrass (Alopecurus myosuroides Huds.)

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Original article

Enhanced activity of several herbicide-degrading enzymes: a suggested mechanism responsible for multiple resistance in blackgrass (*Alopecurus myosuroides* Huds.)

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Abstract – Experiments were carried out to analyse enhanced herbicide metabolism mediated by cytochrome P450 monooxygenases (P450(s)) or glutathione-S transferases (GST(s)) as a possible mechanism responsible for multiple resistance in three blackgrass populations. The RA population is resistant to aryloxyphenoxypropionates (fops) and flupyrsulfuron, the RB to fenoxaprop-P, flupyrsulfuron and ureas and the RC to fenoxaprop-P, haloxyfop and clodinafop. Based on the coleoptile length, growth responses of seedlings to the herbicide responsible for resistance and to combined treatments of the herbicide plus P450(s) or GST(s), inhibitors were compared. Growth of the RA seedlings was significantly reduced with flupyrsulfuron plus malathion, suggesting that P450(s) activity may be involved in the resistance. In contrast, none of the four inhitors tested had an effect on the fop resistance. Fenoxaprop-P plus tridiphane, flupyrsulfuron plus malathion and ureas plus PBO or ABT significantly reduced the RB coleoptiles' growth. Therefore, multiple resistance in this population may be due to an elevation of P450(s) and GST(s) activity. In the RC population, PBO, ABT and tridiphane synergised clodinafop, haloxyfop and fenoxaprop-P effects, suggesting that the two enzymatic systems may also be involved in the RC resistances.

multiple herbicide resistance / enhanced herbicide detoxification / P450(s)/GST(s) / *Alopecurus myosuroides* Huds.

Résumé – L'amplification d'enzymes de détoxication des herbicides : un mécanisme possible responsable des résistances multiples chez le vulpin (*Alopecurus myosuroides* Huds.). Cette expérimentation a pour but d'analyser l'éventuelle implication de monooxygénases à cytochrome P450 (P450(s)) et de glutathione S-transférases (GST(s)) dans les résistances multiples observées chez trois populations de vulpin. La population RA résiste aux aryloxyphenoxypropionates (fops) et au flupyrsulfuron, la RB au fénoxaprop-P, au flupyrsulfuron et aux urées et la RC au fénoxaprop-P, à l'haloxyfop et au clodinafop. Les croissances de la première feuille des plantules exposées à l'herbicide seul et à un mélange "herbicide + inhibiteur" ont été analysées. La croissance des plantules RA est fortement réduite en présence de flupyrsulfuron associé au malathion. Ainsi, des P450(s) pourraient être impliquées dans la résistance de cette population. En revanche, les quatre inhibiteurs sont sans effet sur la résistance aux fops. Comparées à l'effet des herbicides utilisés seuls, les combinaisons fénoxaprop-P plus tridiphane, flupyrsulfuron plus malathion et urées plus PBO ou ABT réduisent significativement la croissance des plantules RB. Ces résultats suggèrent donc que des P450(s) et des GST(s) pourraient être responsables des résistances multiples de cette population. Pour finir, les résistances de la population RC pourraient là aussi résulter de l'activité des deux systèmes enzymatiques puisque la croissance des plantules est significativement inhibée par les herbicides utilisés en association avec l'ABT, le PBO et le tridiphane.

résistances multiples / détoxication / P450(s)/GST(s) / *Alopecurus myosuroides* Huds.

1. INTRODUCTION

The impact of herbicides on our modern agricultural technology has been phenomenal and their usage now exceeds that of fungicides and insecticides combined [18]. The use of herbicides has increased as more selective herbicides have become available and as they replace mechanical tillage as a method of weed control. Nevertheless, an undesirable outcome of widespread use of herbicides for weed control has been the appearance and rapid proliferation of resistant weeds [32]. The 1999 international survey of herbicide-resistant weeds recorded more than 200 resistant biotypes in 45 countries all over the world [18] and herbicide resistance is now posing a major threat to current agricultural practices [20].

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Herbicide resistance can be defined as the natural inherited ability of some biotypes within a population to survive and reproduce following a herbicide treatment that would, under normal conditions of use, effectively control that weed population [18]. A herbicide is a selection agent for any heritable traits within a population that enable survival of individuals in the presence of herbicide. Therefore, the survival mechanism need only be sufficiently efficacious to confer survival at the rate of herbicide used. As herbicide treatments are nearly always at relatively low doses, this ensures survival of individuals expressing both strong and relatively weak resistance mechanisms. Plants with any genetically derived resistance mechanisms will survive and contribute to the subsequent gene pool. When a very large number of a highly variable weed species are treated, there can be survivors with quite different resistance mechanisms [43]. The allogamous nature of a weed species contributes to the development of such multiple resistance. In allogamous species, resistant individuals carrying different resistance genes may cross-pollinate and progeny may then carry both resistance genes. Successive selections may thus accumulate resistance mechanisms within individuals. Multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism, endowing the ability to withstand herbicides from different chemical classes [16]. Multiple resistance is best documented in the weed species *Lolium rigidum* Gaud (rye grass) [4] and, to a small extent, in *Alopecurus myosuroides* Huds. (blackgrass), which exhibit many of the characteristics that favour the accumulation of herbicide resistance mechanisms [3]. These characteristics include a high reproductive capacity, an allogamous reproduction and a genetic plasticity [16]. Such populations that have developed multiple mechanisms, conferring resistance not only to the challenging herbicide but to other herbicide groups, are difficult to control by chemical means. Therefore, elucidation of the mechanisms endowing herbicide multiple resistance in such populations is of prime importance.

Herbicide resistance in weed species may be conferred by two major mechanisms: (I) a mutation of the gene encoding the herbicide target site that confers a high level of resistance, or (II) an enhanced herbicide detoxification by non-specific enzymes that confers a slighter level of resistance [14, 15, 25, 40]. Higher plants are frequently able to metabolise the xenobiotics that they absorb and the phytotoxicity of a herbicide is often closely associated with the rate at which it is metabolised. Most resistant species rapidly metabolise herbicide while susceptible species are unable to do it, or at rates too slow to prevent herbicide from reaching its site of action [23]. Resistance involving herbicide detoxification may be conferred by an elevated expression of cytochrome P450 monooxygenases (P450(s)), a super enzyme family. The existence of P450(s) in higher plants was first proven in the early 1970s [36]. Accumulating evidence indicates that there is a multiplicity of P450(s) in plants [19] and they are best known and most studied for their oxygenase activity [1, 16, 36]. P450(s) have the capacity to attack a vast range of structurally unrelated chemicals to transform them into more polar, soluble products, diminishing or suppressing their toxicity [10]. Another method of detoxification involves the glutathione-S transferases (GST(s)) [12, 23, 24], a group of multiple isozymes which catalyse the conjugation of electrophilic xenobiotics with the tripeptide glutathione (GSH) to form polar, non-toxic peptide conjugates [5, 17]. Consequently, the water solubility, reactivity and biological properties of the electrophile are altered. Conjugation is often followed by sequestration of the conjugate metabolite in a physiologically inactive compartment such as a vacuole [7]. GST(s) are important in detoxifying major classes of herbicides, including chloro-s-triazines, chloroacetanilides, sulfoxide derivatives of thiocarbamates, diphenyl ethers and several aryloxyphenoxypropionates.

In this study, enhanced herbicide metabolism was analysed as a possible mechanism responsible for multiple resistance observed in three blackgrass populations. Inhibitors known to antagonise metabolism-based resistance in plants were used to determine whether resistance in these populations is the result of one or more herbicide-degrading enzymes. Growth responses of seedlings to combined treatments of inhibitors and herbicides responsible for the resistance were analysed.

2. MATERIALS AND METHODS

2.1. Plant material

Three herbicide-resistant populations (RA, RB and RC) and one standard susceptible population (S) of *Alopecrurus myosuroides* Huds. collected in France were used in this study. The RA population is made up of 100% of individuals resistant to three aryloxyphenoxypropionates (fenoxaprop-P, haloxyfop and clodinafop) and flupyrsulfuron, a sulfonylurea herbicide. The RB population is 100% resistant to fenoxaprop P, flupyrsulfuron and two ureas (isoproturon and chlorotoluron). The RC population is made up of 30% of individuals resistant to fenoxaprop-P, haloxyfop and clodinafop. These resistance patterns were first determined using a herbicide laboratory sprayer [27] and then confirmed with a resistance screening bioassay previously developed [28].

2.2. Chemical products

2.2.1. Detoxifying-enzyme Inhibitors

The inhibitors were chosen according to their reported effect on detoxifying enzymes in plants. Piperonyl butoxide (PBO) (Loveland Industries, USA), 1-aminobenzotriazole (ABT) (Sigma, France) and malathion (Cluzeau, France) were used as cytochrome P450 monooxygenase inhibitors [2, 13, 41, 44]. Tridiphane (AGR 280959, Dowelanco, USA) was used as a preferential inhibitor of glutathione-S transferases [23].

To determine for each inhibitor the concentration to use in combination with each herbicide, we selected from among a range of doses the highest one that had no phytotoxic effect on its own on the coleoptile growth of the susceptible seedlings. Inhibitor solutions containing 2.5, 5, 10, 20, 40 and 80 $mg \cdot L^{-1}$ ABT, PBO, malathion and 0.15625, 0.3125, 0.625, 1.25, 2.5 and 5 $mg \cdot L^{-1}$ tridiphane were prepared by serial dilutions in distilled water.

2.2.2. Herbicides

The experiments were carried out with the acid or technical forms of the herbicides rather than the commercial formulations. The advantage of these forms compared with the commercial formulations is a higher solubility in water (e.g.: fenoxaprop-P acid, 61 $g \cdot L^{-1}$; fenoxaprop-P-P-ethyl, 0.7 $mg \cdot L^{-1}$). Therefore, these forms allow preparation of "true" aqueous solutions of active ingredients.

The herbicide concentrations were previously determined as reliable concentrations to discriminate between the resistant and susceptible individuals with a seedling bioassay based on the coleoptile length (28, 29, 31 and see below). Herbicide solutions containing 6 $mg \cdot L^{-1}$ fenoxaprop-P acid, 2.5 $mg \cdot L^{-1}$ haloxyfop acid (AgrEvo, Germany), 5 $mg \cdot L^{-1}$ clodinafop propargyl (Novartis, France), 6 $mg \cdot L^{-1}$ flupyrsulfuron (Dupont, France) and 8 $mg \cdot L^{-1}$ isoproturon and chlorotoluron (Aventis, France) were prepared by serial dilutions. Each herbicide had been dissolved previously in 0.1% (V/V) dimethylsulphoxide (DMSO). The pH of the herbicide solutions was adjusted to pH 7 with a Tris[hydroxymethyl]aminomethane (Tris) buffer.

2.3. Dose-response experiments

Except for ureas, the herbicide and inhibitor effects were determined using the herbicide resistance screening bioassay previously developed and based on the coleoptile length of pre-germinated seeds exposed for six days to the discriminating herbicide concentration that provided the largest difference in coleoptile length of the susceptible and resistant seedlings [28]. According to this seedling test, the lengths of the susceptible (S) and resistant (R) coleoptiles were always shorter and longer than 10 mm long, respectively. As isoproturon and chlorotoluron inhibit photosynthesis, it was shown that coleoptile length was not the best discriminative parameter for assessing resistance to ureas [28, 29]. Thus, for these two herbicides, resistance was screened based on the complete bleaching of the coleoptiles within two weeks. At the discriminating concentration selected, all the S coleoptiles were bleached while the R ones were still green like the control [26, 28].

To conduct all the dose-response experiments, seeds of each population 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 $g \cdot m^{-2}$, Müller). To stimulate seed germination, the petri-dishes were filled with 30 mL of 2 g $KNO_3 \cdot L^{-1}$ and placed in a controlled environment room (12 h, 20 °C light/12 h, 15 °C dark). After 4 days, pre-germinated seeds with rootlets 3–4 mm long were transferred onto one sheet of blotting paper (Germaflor No. 55, 160 $g \cdot m^{-2}$, Müller) laid on two layers of 6-mm-diameter glass marbles in a plastic box (9 cm × 9 cm × 9 cm). Each box was filled with 25 mL growth medium, closed and placed for six days in a controlled environment room (18 h, 22 °C light/6 h, 20 °C dark).

Pre-germinated seeds of the three resistant populations (RA, RB and RC) were exposed to a control medium (without herbicide and inhibitor), to the selective dose of each herbicide responsible for the resistance and to all the combinations of herbicide plus inhibitor. For all experiments, 100 pre-germinated seeds were used (25 seeds per box). Except for the two ureas, the herbicide and inhibitor effects were assessed after six days' growth by measuring the coleoptile length (from the point of seed attachment to the coleoptile tip). For ureas we estimated, two weeks later, the degree of bleaching.

The inhibitor concentration used in association with the herbicide was the highest one among a range of doses that had no phytotoxic effect on its own. Then, when the growth (or colouring) of a resistant coleoptile was affected by a combination of herbicide plus inhibitor, it only resulted from the inhibition of the herbicide detoxifying enzyme. To determine the reliable concentration of each inhibitor, 100 pre-germinated seeds of the S population (25 seeds per box) were exposed to the range of doses (see above). From among the range of inhibitor doses tested, we selected the highest one that still allowed a coleoptile growth at least equal to 95% of the control.

The effect of each treatment was assessed by measuring the coleoptile length, which was expressed as a percentage of the control.

The control medium was made of distilled water and the pH was adjusted to pH 7 with a Tris buffer.

2.4. Statistical analysis

For each experiment, data were subjected to an analysis of variance (ANOVA) and the mean coleoptile lengths were separated with a Duncan's multiple range test ($P < 0.05$) [37].

3. RESULTS

3.1. Dose-response to the herbicides

When the S seedlings were exposed for six days to the three aryloxyphenoxypropionate herbicides and flupyrsulfuron, they were strongly inhibited by the concentration selected, as they were shorter than 5 mm (Fig. 1A). According to the seedling bioassay, none of the RA coleoptiles were inhibited by the herbicides tested, compared with the S coleoptiles, and they were always longer than 10 mm (Fig. 1B). In the same way, all the RB coleoptiles were longer than 10 mm in the presence of the discriminating dose of fenoxaprop-P and flupyrsulfuron. In contrast, they were strongly inhibited by clodinafop and haloxyfop (4 and 3 mm long, respectively) (Fig. 1C). When exposed to the three aryloxyphenoxypropionate herbicides some RC coleoptiles were always longer than 10 mm (Fig. 1D). For this population, about 30% of seedlings were longer than 10 mm in the presence of these three herbicides (results not shown). In contrast, all the RC coleoptiles were strongly inhibited by flupyrsulfuron (Fig. 1D). In the presence of the two ureas, all the S and R coleoptiles tested were bleached, except for the RB coleoptiles (Tab. I).

Therefore, according to the seedling bioassay previously developed (see materials and methods section), the RA population was resistant to the three aryloxyphenoxypropionates and flupyrsulfuron, the RB population was resistant to fenoxaprop-P, flupyrsulfuron and ureas and the RC population was resistant to the three aryloxyphenoxypropionates. All these results confirm what is observed in the field.

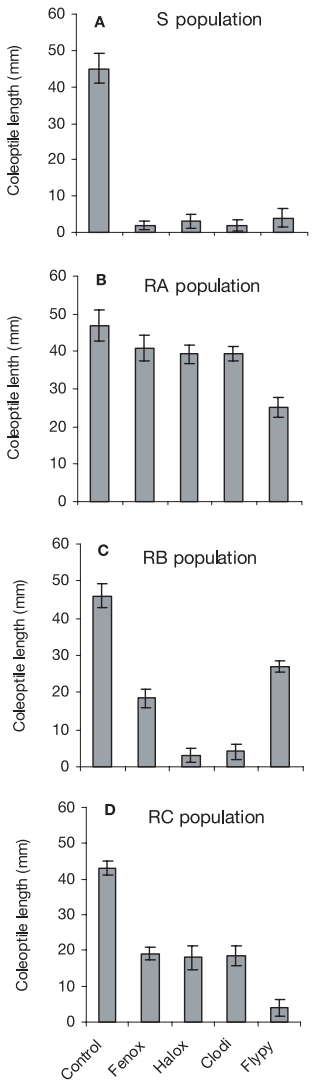

Figure 1. Mean coleoptile length of 100 seedlings for the four blackgrass populations (S, RA, RB and RC) exposed for six days to 6 mg·L^{-1} fenoxaprop-P acid (Fenox), 2.5 mg·L^{-1} haloxyfop acid (Halox), 5 mg·L^{-1} Clodinafop propargyl (Clodi) and 6 mg·L^{-1} flupyrsulfuron (Flupy). S: susceptible – R: resistant. Vertical bars = +/– standard errors.

3.2. Dose-response to the associations herbicide plus inhibitor

3.2.1. RA population

The combined effects of the herbicides responsible for resistance in the RA population and the four inhibitors on the coleoptile length of the seedlings are presented in Figure 2. For fenoxaprop-P, haloxyfop and clodinafop, there were no significant differences between the mean coleoptile lengths of the RA seedlings exposed to the herbicide alone or combined with each inhibitor (Fig. 2A, B and C). In the same way, when compared with the effect of 6 mg·L^{-1} flupyrsulfuron alone, combinations of this herbicide with ABT, PBO or tridiphane did not significantly reduce the RA coleoptiles' length. In contrast, significant reduction of the RA coleoptiles was recorded when flupyrsulfuron was combined with malathion (Fig. 2D). While the RA coleoptiles exposed to flupyrsulfuron were 25 mm long, they were shorter than 5 mm when exposed to the association flupyrsulfuron plus malathion.

Table I. Percentage of bleached coleoptiles for the four blackgrass (S, RA, RB and RC) exposed for 2 weeks in 8 mg·L^{-1} isoproturon and chlorotoluron. One hundred coleoptiles were tested per population. S: susceptible – R: resistant.

Herbicide	Populations	% of bleached coleoptiles
Isoproturon	S	100
	RA	100
	RB	0
	RC	100
Chlorotoluron	S	100
	RA	100
	RB	0
	RC	100

3.2.2. RB population

Addition of ABT, PBO and malathion to fenoxaprop-P did not significantly affect the RB coleoptiles' growth compared with what was observed in the presence of the herbicide alone. In contrast, the combination of fenoxaprop-P plus tridiphane strongly reduced the RB coleoptiles (Fig. 3A). In the presence of fenoxaprop-P, the RB coleoptiles were still 18 mm long, whereas they did not exceed 6 mm with fenoxaprop-P plus tridiphane. For flupyrsulfuron, the addition of ABT, PBO and tridiphane did not significantly reduce the RB coleoptiles' mean length compared with the length measured in the presence of flupyrsulfuron alone (Fig. 3B). In contrast, the addition of malathion to the herbicide solution was significantly effective and strongly inhibited the coleoptile growth of all the RB seedlings, which never exceeded 3.5 mm in length (Fig. 3B). For isoproturon and chlorotoluron, Table II shows that the addition of ABT and PBO to the herbicide solution induced bleaching of the RB coleoptiles, whereas malathion and tridiphane were without effect on the large majority of the coleoptiles.

3.2.3. RC population

For population C, when compared with the effect of fenoxaprop-P alone, combination with ABT, PBO or malathion did not significantly reduce the length of the R coleoptiles (Fig. 4A). Among the 100 seedlings tested, 28 were not affected (results not shown). In contrast, the addition of tridiphane to the herbicide solution strongly inhibited the growth of all the RB coleoptiles compared with what was observed when seedlings were exposed to fenoxaprop-P alone (Fig. 4A). Indeed, with fenoxaprop-P the RC coleoptiles that were not affected were still 19 mm long, while they were all shorter than 5 mm with the combination. Compared with haloxyfop alone, the RC coleoptiles' growth was significantly inhibited by both ABT and PBO, while malathion and tridiphane were not effective on the R coleoptiles' growth (Fig. 4B). Indeed, these two last inhibitors were without any

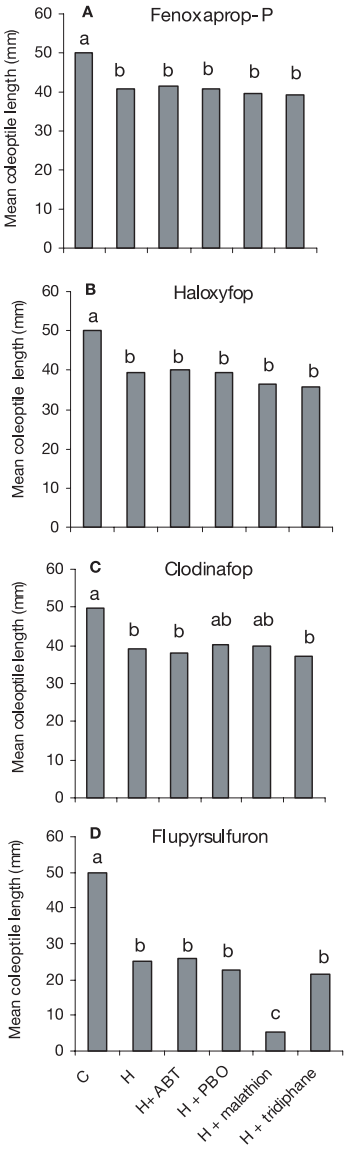

Figure 2. Mean coleoptile length of 100 RA seedlings exposed for six days to 6 mg·L^{-1}, 2.5 mg·L^{-1} haloxyfop, 5 mg·L^{-1} diclofop and 6 mg·L^{-1} flupyrsulfuron alone or in combination with the four detoxifying-enzyme inhibitors. C: control, H: herbicide, ABT: 1-aminobenzotriazole, PBO: piperonyl-butoxide. Different letters indicate significant difference between coleoptile lengths (Duncan's multiple range test at the 0.05 level).

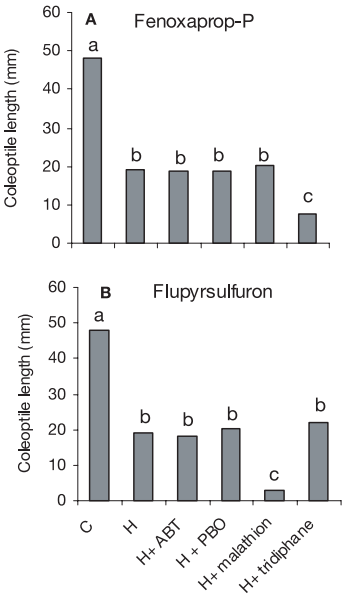

Figure 3. Mean coleoptile length of 100 RB seedlings exposed for six days to 6 mg·L^{-1} and flupyrsulfuron alone or in combination with the four detoxifying-enzyme inhibitors. C: control, H: herbicide, ABT: 1-aminobenzotriazole, PBO: piperonyl-butoxide. Different letters indicate significant difference between coleoptile lengths (Duncan's multiple range test at the 0.05 level).

Table II. Percentage of bleached coleoptiles for the RB population exposed for 2 weeks to 8 mg·L^{-1} isoproturon and chlorotoluron alone or in combination with the four detoxifying-enzyme inhibitors. One hundred coleoptiles were tested per treatment. ABT: a-aminobenzotriazole, PBO: piperonyl-butoxide.

Herbicide	Inhibitor	% of bleached coleoptiles
Isoproturon	-	0
	ABT	100
	PBO	100
	malathion	2
	tridiphane	1
Chlorotoluron	-	0
	ABT	100
	PBO	100
	malathion	1
	tridiphane	1

effect on 30 RB seedlings among the 100 seedlings tested (result not shown). In the same way, combinations of clodinafop with ABT or PBO significantly reduced all the RC coleoptiles compared with what was observed with the herbicide alone. As for haloxyfop, the two other combinations did not cause a significant reduction in the coleoptile length compared with the herbicide alone (Fig. 4C). Still, 30 RC seedlings were not affected by these combinations (results not shown).

All these results are summarised in Table III.

4. DISCUSSION

This experiment was conducted to analyse whether enhanced herbicide metabolism was a possible mechanism responsible for multiple resistance in the three blackgrass populations studied. Four inhibitors, ABT, PBO, malathion and tridiphane, known to antagonise metabolism-based resistance by inhibiting herbicide-detoxifying enzymes such as P450(s) and GST(s), were used in combination with the herbicides responsible for resistance.

In addition to their role in herbicide detoxification, P450(s) and GST(s) are also involved in important physiological

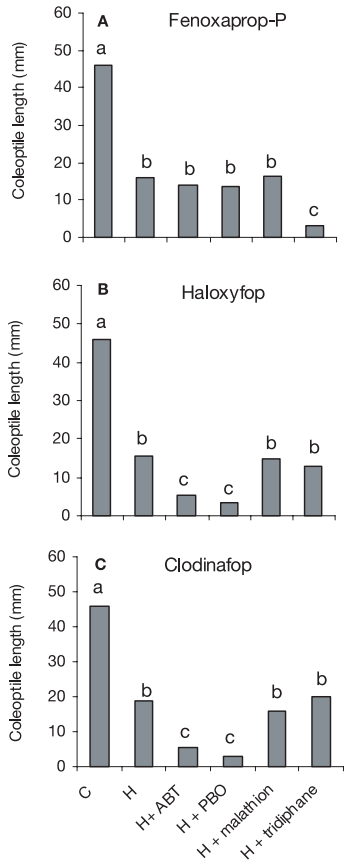

Figure 4. Mean coleoptile length of 100 RC seedlings exposed for six days to 6 mg·L^{-1}, 2.5 mg·L^{-1} and 5 mg·L^{-1} clodinafop alone or in combination with the four detoxifying-enzyme inhibitors. C: control, H: herbicide, ABT: 1-aminobenzotriazole, PBO: piperonyl-butoxide. Different letters indicate significant difference between coleoptile lengths (Duncan's multiple range test at the 0.05 level).

reactions in plants such as protection during oxidative stress, transport and regulation of certain metabolites or detoxification of endogenous compounds [33]. This is why increasing concentrations of each inhibitor used alone are not without effect on S coleoptiles' growth (results not shown). Thus, for each inhibitor we selected the highest concentration that did not reduce coleoptile growth (see Material and Methods).

The results shown in Figure 2 indicate that the RA seedlings became susceptible to flupyrsulfuron when malathion was used. As malathion is described as a potential P450(s) inhibitor [33], these results suggest that P450(s) activity may be responsible for flupyrsulfuron resistance in the RA population. This result is consistent with a previous study where malathion was shown to synergise the action of another sulfonylurea herbicide, chlorsulfuron, in *Lolium rigidum* [33]. This biochemical study revealed that malathion increased chlorsulfuron toxicity by inhibiting the herbicide metabolism mediated by P450(s). On the other hand, none of the four inhibitors tested affected the coleoptile growth when the three aryloxyphenoxypropionates were used (Fig. 2). Thus, we may suppose that

Table III. Summary of the action of the four inhibitors on herbicide effects in the three resistant blackgrass populations. Cross means that the inhibitors synergises the herbicide effect. ABT: a-aminobenzotriazole, PBO: piperonyl-butoxide. R: resistant.

Populations	Herbicides	P450(s) inhibitors			GST(s) inhibitor
		ABT	PBO	malathion	tridiphane
RA	Fenoxaprop-P				
	Haloxyfop				
	Clodinafop propargyl				
	Flupysulfuron			X	
RB	Fenoxaprop-P				X
	Isoproturon	X	X		
	Chloroproturon	X	X		
	Flupysulfuron			X	
RC	Fenoxaprop-P				X
	Haloxyfop	X	X		
	Clodinafop propargyl	X	X		

either none of the herbicide-detoxifying enzymes investigated in this study are responsible for resistance to aryloxyphenoxypropionates of the RA population, or another mechanism is involved in the resistance. A previous study, conducted with the same blackgrass population, had suggested that this population may contain a mutated acetyl CoA carboxylase (ACCAse) [3, 30], the target-site of aryloxyphenoxypropionates [22, 35, 38, 40]. Target site-based resistance is the most commonly reported mechanism of resistance to ACCase-inhibiting herbicides [9, 39]. Therefore, in addition to possessing a probably resistant ACCase, the RA biotype may show an enhanced metabolism of flupyrsulfuron, endowed by P450(s). As 100% of the RA seedlings are resistant to the three aryloxyphenoxypropionates and to flupyrsulfuron, we may speculate that all the RA seedlings may possess both a mutated ACCase and an increased activity of P450(s) susceptible to malathion.

Moreover, an experiment conducted to investigate the inheritance of fenoxaprop-P resistance within the RA population has suggested that the RA individuals may also resist this herbicide due to an enhanced activity of P450(s) inhibited by malathion [31]. This mechanism of resistance to fenoxaprop-P was identified thanks to the segregation genes in the F2 generation. But here, as we only investigated the mechanism of fenoxaprop-P resistance within the F1 generation, the possible implication of P450(s) susceptible to malathion in the resistance could not be identified because it may be disguised by the presence of a mutated ACCase.

Fenoxaprop-P phytotoxicity was increased in the RB biotype in the presence of tridiphane (Fig. 3), a potential GST inhibitor [23]. Thus, this result suggests that increased activity of GST(s) may be responsible for fenoxaprop-P degradation and therefore for herbicide resistance in this population. Glutathione conjugation was first suggested as a possible pathway of fenoxaprop-p detoxification in barley, crabgrass, oat and wheat [11, 42]. These in vitro metabolism studies revealed that

fenoxaprop-P underwent rapid displacement of the phenyl group by glutathione (GSH). In blackgrass, the involvement of GST in fenoxaprop-P resistance was first demonstrated in the English population "Peldon" where the GST activity was significantly increased in the presence of the herbicide [7, 34]. The role of GST(s) in resistance to multiple herbicides in blackgrass was more recently confirmed by Cummins et al. [8]. This work has suggested that GST(s) may function as glutathione peroxiades to prevent oxidative injury caused as a primary or secondary effect of herbicide action.

Malathion synergised the effect of flupyrsulfuron on RB seedlings, whereas PBO and ABT synergised the effects of isoproturon and chlorotoluron (Fig. 3 and Tab. II). Malathion, ABT and PBO are all P450 inhibitors. Therefore, we may suppose that the RB seedlings are resistant to flupyrsulfuron and ureas, probably due to an increased activity of two different isoenzymes of P450(s). A previous study conducted to investigate chlorotoluron metabolism in leaves of resistant and susceptible biotypes of blackgrass demonstrated the implication of P450(s) in the resistance to ureas [21]. According to our results, we may therefore speculate that at least three different herbicide-metabolising enzymes have increased activity in the RB population: GST(s), P450(s) inhibited by malathion and P450(s) affected by both ABT and PBO. Moreover, as the entire population is resistant to fenoxaprop-P, flupyrsulfuron and ureas, all the seedlings could show an increased activity of the three enzymatic systems. We suggest that malathion inhibits the P450s responsible for metabolising flupyrsulfuron, but not those responsible for metabolising urea herbicides. In contrast, PBO and ABT inhibit the P450s responsible for metabolising urea herbicides, but not flupyrsulfuron. Thus, even though the large majority of the P450(s) are known to show a broad and partially overlapping substrate specificity [6], this experiment revealed that some of them might be specific for one herbicide family.

As for the RB population, tridiphane antagonises fenoxaprop-P resistance in the RC population, suggesting that an enhanced activity of GST(s) may also be involved in this resistance (Fig. 4). Figure 4 also shows that the RC seedlings became susceptible to haloxyfop and clodinafop in the presence of ABT and PBO. Thus, in addition to the GST activity, the RC population may show an enhanced activity of P450(s). Contrary to the RA and RB populations, 100% resistant, the RC population is only made up of about 30% resistant individuals to fenoxaprop-P, haloxyfop and clodinafop (results not shown). Thus, either the same 30% of plants may show an enhanced activity of both GST(s) and P450(s) or either 30% possess an amplification of GST(s) and another 30% an amplification of P450(s). As blackgrass is an allogamous species, the most probable hypothesis is that some resistant individuals effectively show an enhanced activity of both GST(s) and P450(s) while some others may only possess an amplification of one of the two enzymatic systems.

Based on both the differential patterns of herbicide resistance and inhibition of herbicide metabolism by cytochrome P450 inhibitors (Tab. III), we may suggest that at least three metabolic systems involving different isoenzymes of cytochrome P450 monooxygenases may contribute to enhanced detoxification in blackgrass. At least one isoenzyme might be responsible for resistance to flupyrsulfuron, a second for resistance to haloxyfop and clodinafop and a third isoenzyme may give enhanced metabolism of ureas. While malathion appears to be specific for the P450(s) involved in flupyrsulfuron degradation, this study revealed that ABT and PBO may possess a broad spectrum of substrates, as they both inhibited at least two different isoenzymes of P450(s): one responsible for the detoxification of the two ureas and another responsible for detoxification of haloxyfop and clodinafop. Moreover, we cannot exclude the hypothesis that three or even four isoenzymes of P450(s) may be specifically involved in the detoxification of these four herbicides, all inhibited by ABT and PBO.

Even though the rigorous proof of involvement of detoxifying enzymes in the resistance responses requires the detection of the catalysed herbicide breakdown products and the demonstration that the biotypes have different capacities to produce these compounds, this study strongly suggests that the multiple resistance observed in the three blackgrass populations may be the result of an enhanced metabolism of herbicide involving P450(s) and, more rarely, GST(s). Moreover, this work shows the possible accumulation of different resistance mechanisms in a single population and even in a single plant. This phenomena has already been observed in rye grass populations [33] and in the resistant blackgrass population from the UK, Peldon [3]. The out-crossing nature of blackgrass in combination with the extensive and varied herbicide applications provide ideal conditions for accumulation of multiple resistance mechanisms. In addition, it appears that populations such as the ones studied in this experiment would be resistant to herbicide families yet to be marketed or even discovered, particularly where such herbicide chemistries can be metabolised by P450(s). While blackgrass and rye grass share common biological elements, they are not unique. Multiple herbicide resistance is predicted to develop in other weed species. This phenomena 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 to be the most effective resistance management strategy.

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