POSTER SESSION 3C

HERBICIDE RESISTANCE: MECHANISMS AND DIAGNOSTICS

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Foster Papers: 3C-1 to 3C-9

Evaluation of *Lolium rigidum* biotypes resistance to chlorsulfuron: useful parameters

N de la Carrera, M Villarroya, M C Chueca; J M Garcia-Baudin
Dpto Proteccion Vegetal. INIA Carretera La Coruña km 7.5 Madrid, Spain

ABSTRACT

Parameters such as plant kill, growth stage and length of the second and third leaf enable detection of biotypes resistant and susceptible to chlorsulfuron. These parameters were used to study three biotypes of *Lolium rigidum*. One of the biotypes was collected in a wheat field in the region of the Duero river, where poor control of *L. rigidum* with chlorsulfuron had been observed. The response of this biotype was compared to control with chlorsulfuron of reference resistant and susceptible biotypes. The biotype studied presents a resistant response, although with a lower level of resistance that the resistant biotype.

INTRODUCTION

The widespread use of herbicides for weed control over the past decades has exposed huge weed populations to strong selection pressures that lead to the appearance and proliferation of weeds resistant to different chemical classes of herbicides. *Lolium rigidum* is one of the most serious problems among weeds that have evolved herbicide resistance (Le Baron, 1991). In Australia numerous cases of *L. rigidum* resistance to herbicides have been reported (Matthews, 1994). This species has evolved resistance to more than 16 chemical classes of herbicides (Holt *et al.*, 1993) and one population can be resistant to herbicides belonging to different modes of action even without prior exposure to these herbicides (Preston *et al.*, 1996). The most frequent cases of herbicide resistance results from changes in the herbicide target enzyme.

Sulfonylurea herbicides were introduced commercially in 1982. Since this time, and in a relatively short period, resistance to these herbicides has become increasingly frequent in many weed species. Chlorsulfuron is a sulfonylurea herbicide that inhibits acetolactate synthase (ALS) a key enzyme in the synthesis of the branched chain amino acids. Resistance to these herbicides by an alteration of the target enzyme (Ray, 1984) and by metabolism (Hutchinson *et al.*, 1984) have been detected.

L. rigidum is one of the most abundant grass weeds in cereals (García-Baudín, 1982). Fernandez (1998) has detected a lack of its control in cereal fields in the Duero region where 56% of over 300 farmers surveyed cite it as being abundant. This paper presents the response of three biotypes of rigid ryegrass to chlorsulfuron using three parameters to assess resistance.

MATERIALS AND METHODS

The three biotypes of *Lolium rigidum* used were AUS97 and Stein resistant and susceptible to sulfonylurea herbicides respectively (provided by Dr Boutsalis) and one (MT) collected in wheat field in Zamora (Duero region) where declining herbicide efficacy was detected.

Seeds were germinated in petri dishes containing filter paper moistened with water for four days. Seedlings were planted at one seed per pot in a (1:2) sand:soil mixture (pH=7±0.5, EC=290 µmhos/cm) contained in 75 ml plastic pots. Plants were treated 24 hours after transplanting with a comercial formulation of chlorsulfuron (75% a.i.) at the rates of 0, 10, 20, and 40 g/ha of comercial product. Herbicide applications were made with a laboratory sprayer equipped with a flat–fan nozzle (Teejet 8003) at 220 kPa.

Plants were grown in a growth chamber with a 16 hour photoperiod and $100\mu Em^{-2}s^{-1}$. Day and night temperatures were 21±1 and 18±1 °C respectively.

Assessments were made of mortality of plants 15, 30 and 45 days after treatment (plants were scored as dead if they had no green tissue remaining), growth stage (number of leaves) 15 and 30 days after treatment and length of the second and third leaf 30 days after treatment.

All the assays were carried out with 100 plants for each treatment and dose and replicated twice in time. The response of the three lines followed the same pattern, although there were differences between experiments. A non parametric test (Kruskal-Wallis) was used to compare the distribution of second and third leaves in the three lines studied. A chi-square test was used to compare the frequencies of leaves.

RESULTS AND DISCUSSION

The three biotypes of *L. rigidum* show different responses to chlorsulfuron (Table 1). This is a herbicide with a slow action and therefore there is no appreciable mortality of plants 15 days after treatment. However, Stein biotype begins to show an increase in plant mortality thirty days after the treatment, reaching 46% for the highest dose used and 62% 45 days after treatment. The AUS97 and MT biotypes have a low mortality with this same dose with 83 and 81% survival respectively 30 days after treatment and 78 and 60% 45 days after treatment.

Figure 1 shows the stage of growth of the three biotypes as judged by emergence of successive leaves. Fifteen days after spraying, 71% of the untreated control plants of the Stein susceptible biotype have two leaves and 29% have three leaves. Following treatment with 40g/ha, 72% of the plants that were still alive remained at the one leaf stage and only 28% have two or three leaves. With the AUS97 and MT biotype, both control and treated plants had developed the same number of leaves, three for AUS97 and two for MT, which provides evidence of more rapid early development of AUS 97. Thirty days after treatment, a different response is evident for each of the biotypes. Most untreated control plants of all the biotypes had reached the four leaf growth stage. In the susceptible biotype, 40% of the living plants were still at the one leaf stage following treatment with the highest dose of chlorsulfuron.

Table 1. Survival of *L. rigidum* biotypes after treatment with chorsulfuron.

Biotype	Dose (g/ha) commercial product	% Surviving plants		
		15 days after treatment	30 days after treatment	45 days after treatment
Stein	0	90	90	90
	10	92	68	68
	20	96	69	56
	40	80	54	38
Aus97	0	98	97	97
	10	92	91	87
	20	92	86	81
	40	97	83	78
MT	0	98	98	97
	10	99	97	91
	20	100	87	74
	40	99	81	60

Both the untreated and sprayed plants of the biotype used as a standard for resistance had developed four leaves, while in the MT biotype, the response can be considered intermediate between Stein and AUS97 with 61% of the treated plants at the same stage of development as the untreated plants even at the lowest dose. MT show a response significatly different from AUS 97 and from Stein (χ^2 contingency test α= 0.05).

Figure 2 shows the distribution of leaf length for the second and third leaves 30 days after treatment. Generally, the length of the leaves of the control plants is greater than those of treated plants, even in the resistant biotype, in which some of the plants failed to develop the second or third leaf. The most common leaf lengths in control plants for Stein, is 10-15 cm and 20-25 cm for the second and third leaves respectively. In the plants treated with 20 and 40 g/ha the second leaf is usually absent and following application of 10 g/ha most leaves are less than 5 cm in length. Similar trends following treatment with chlorsulfuron are seen for the third leaf. In the AUS97 resistant biotype, a slight reduction in the size of the leaves is observed in the treated plants while for the MT biotype shortening of the second and third leaf is more pronounced and the vigour of the plants of the MT biotype is affected by all doses of the herbicide. The MT response for length of second and third leaf was in between the AUS 97 and the Stein response being significantly different from both (Kruskal-Wallis α= 0.05).

These results show that the parameters we measured enabled clear distinction between rigid ryegrass biotypes resistant and susceptible to chlorsulfuron. From the study of the three

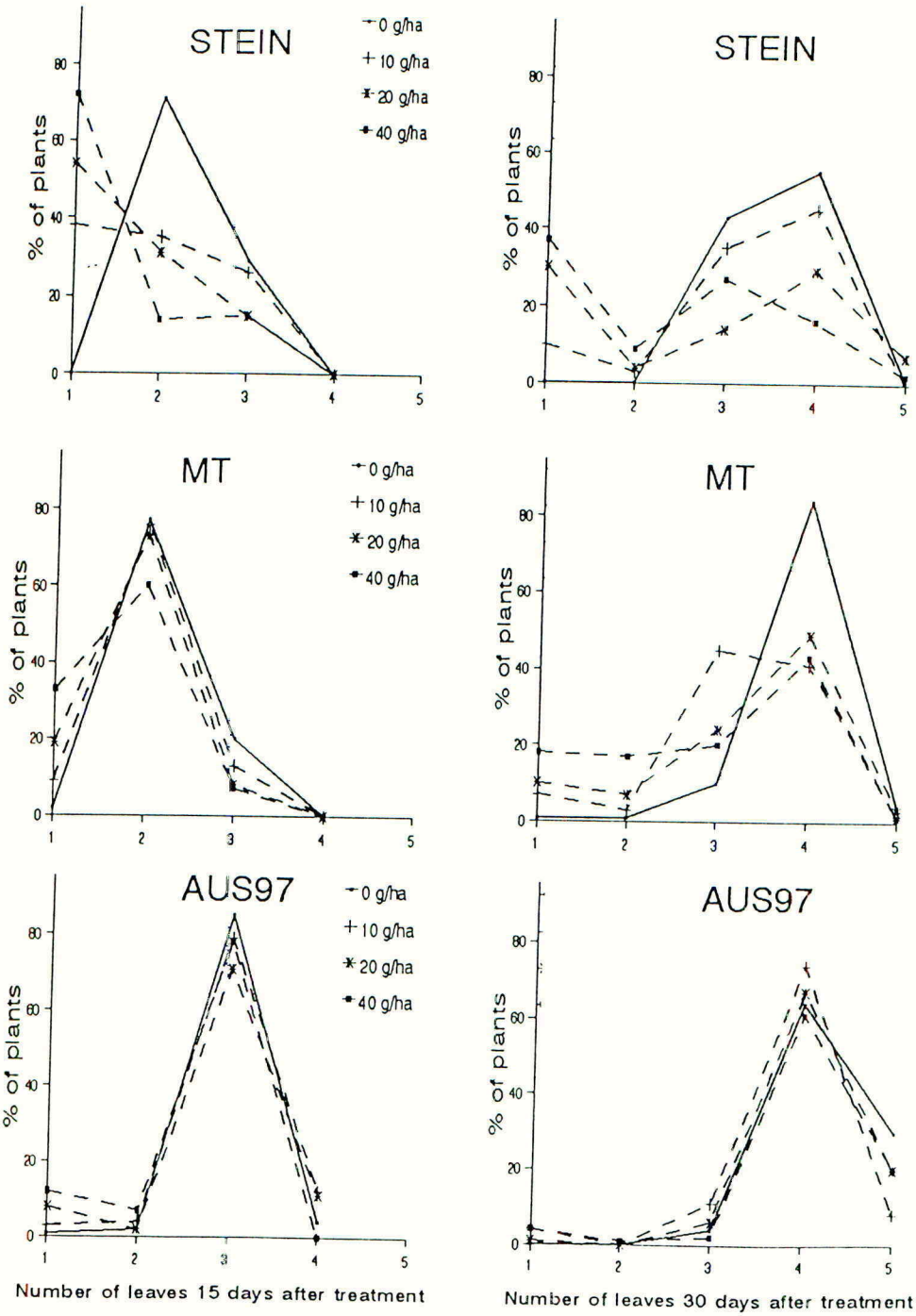

Figure 1. Growth stage of three *Lolium rigidum* biotypes 15 and 30 days after chlorsulfuron treatment.

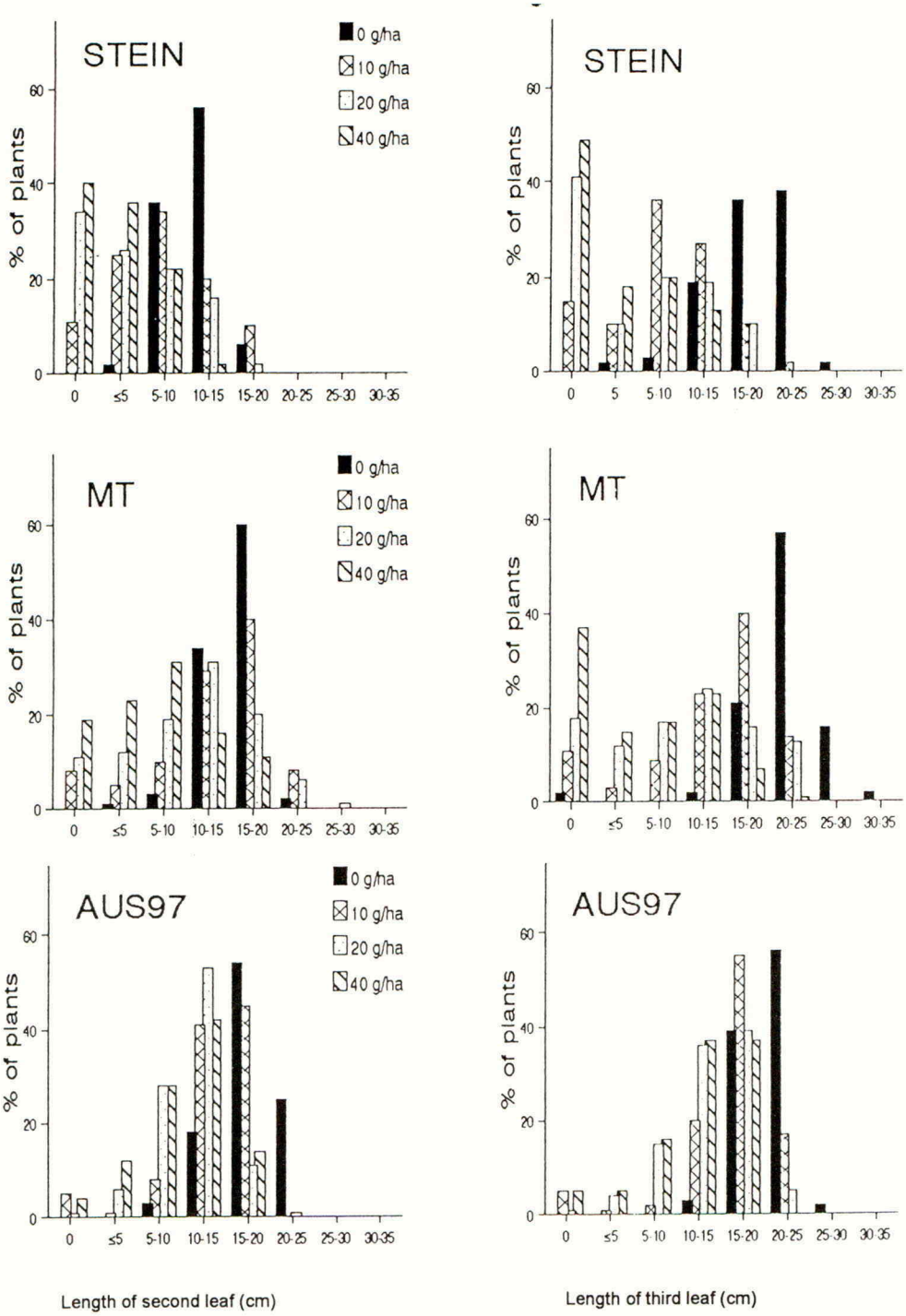

Figure 2. Distribution of second and third leaf length of three *Lolium rigidum* biotypes 30 days after chlorosulfuron treatment.

biotypes we can conclude that the survival of the MT biotype in cereal fields treated with chlorsulfuron is due to resistance to this herbicide. The results also show that MT is less resistant than the AUS97 which is a reference chlorsulfuron resistant biotype. Although MT is slightly less vigourous that AUS97 it establishes well in cereal fields and this and other resistant biotypes are increasingly being reported in the Duero region of Spain. We consider this to be a serious and increasing problem made worse by intensive use of sulfonylurea herbicides with their use on almost 45% of the area infested with rigid ryegrass (Fernandez, 1998).

Although most cases of resistance to sulfonylurea detected in *L. rigidum* are due to target site alteration, the MT biotype has a lower level of resistant than the reference resistant biotype AUS97 and we cannot rule out the possibility that this resistance could be due to metabolic inactivation or enhanced detoxification since the selectivity of certain crops, such as wheat and various weeds is due to the metabolization of the herbicide (Sweetser *et al.*, 1982; Hutchison *et al.*, 1984).

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Dose response curves of resistant and susceptible *Bidens pilosa* to ALS inhibitor herbicides

P J Christoffoleti
Universidade de São Paulo, ESALQ, Caixa Postal 09, CEP 13418-900, Piracicaba, SP, Brazil

L L Foloni
Universidade de Campinas, Fac. de Engenharia, C. P. 6011, CEP 13001, Campinas, SP, Brazil

ABSTRACT

Herbicide resistant weeds have been found in soybean fields in Brazil since 1995, however, identification of resistance was restricted to field tests limited to survival of *Bidens pilosa* following application of recommended field doses. Therefore, an experiment was conducted under greenhouse conditions in order to compare the dose response curves of a resistant (R) and susceptible (S) *Bidens pilosa* biotype to acetolactate synthase (ALS) inhibitor herbicides. Seeds of *B. pilosa* from a suspected resistant population and those from an area that had never been sprayed with these herbicides were used in a pot experiment. At the two to three pair of leaves growth stage plants were sprayed with the herbicides at rates of 0.0; 0.001, 0.01, 0.1; 1.0; 10; 100 and 1000 fold the recommended rate sprayed in soybean fields (chlorymuron-ethyl, imazethapyr), corn fields (nicosulfuron) or wheat fields (metsulfuron-methyl). Adjustments of the dose response curves was done using a log-logistic model. The GR_{50} R/S relation were 85; 120; 250 and 2.5 for the herbicides chlorimuron-ethyl, metsulfuron-methyl, nicosulfuron and imazethapyr respectively. It was concluded that the R *B. pilosa* biotype has a high degree of cross resistance to sulfonylurea and imidazolinone herbicides.

INTRODUCTION

Weed resistance to acetolactate synthase inhibitor herbicides was first reported in Brazil in 1995. One important weed associated with soybean production areas is *Bidens pilosa.* Populations of this weed have survived recommended doses of acetolactate synthase inhibitor herbicides in field tests (Christoffoleti *et al.*, 1996; Ponchio *et al.*, 1996). A more reliable way to confirm resistance is to use dose responses.

The model described by Streibig *et al.* (1993) is widely used and the adoption by Seefeldt *et al.* (1995) was chosen for this study. The main advantage of the log-logistic model is the term that is part of the equation called GR_{50}, that is the rate necessary to control 50 % of the population.

Christoffoleti *et al.* (1996) reported the first use of GR_{50} for confirmation of herbicide resistant biotype in Brazil. Several experiments were conducted in greenhouse conditions,

using resistant and susceptible biotypes of *Bidens pilosa*, and increasing rates of ALS inhibitor herbicides. The GR_{50} was determined for the herbicides imazethapyr, nicosulfuron, metsulfuron and chlorimuron, were 370, 39, 26 and 12 times higher for the resistant biotype, compared to the susceptible one.

The objective of this research was to characterize the resistance level to ALS inhibitor herbicide of another *Bidens pilosa* biotype from soybean fields in greenhouse conditions, using dose response curves.

MATERIAL AND METHODS

Seeds of resistant *Bidens pilosa* were obtained from a field located in São Gabriel do Oeste County, Mato Grosso do Sul State, Brazil, which had been sprayed with ALS inhibitor herbicides for more than eight years. The susceptible seed source was an area in an experimental field of University of São Paulo, located in Piracicaba County, São Paulo State, that had never been sprayed with ALS inhibitor herbicides.

The experiment was set up in the greenhouse where the average temperature was about 27°C. Three litre capacity pots containing a substrate of one part sand, one part vermiculite and one part soil, were planted with ten seeds and after emergence thinned to four plants. At the three to four leaf stage the plants were sprayed using a laboratory sprayer fitted with a 11001E nozzle and calibrated to deliver 200 litres/ha. Application was made at 0.0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 times the field rates of the herbicides chlorimuron-ethyl (20 g a.i./ha), nicosulfuron (5 g a.i./ha), metsulfuron (3 g a.i./ha) and imazethapyr (100 g a.i./ha).

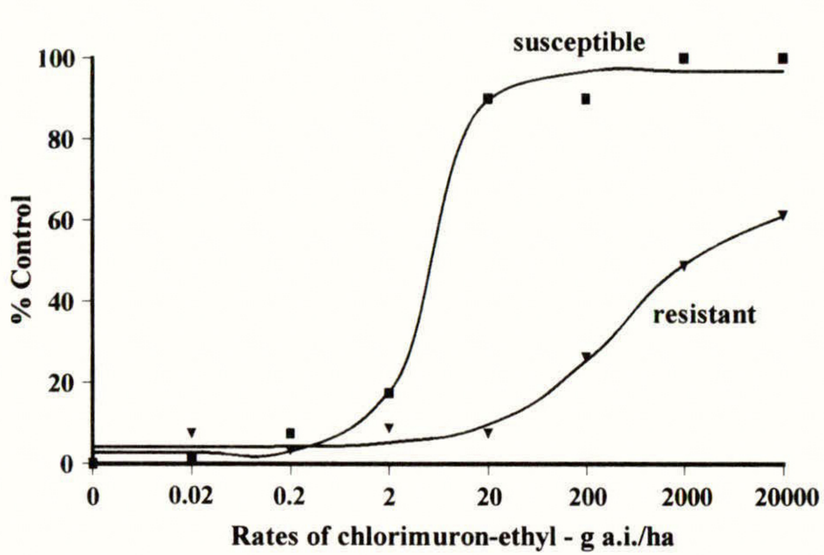
There were six replicate pots which were arranged in a randomized complete design. Twenty-three days after spraying visual assessment was made. The data was presented as percent control and fitted to the non linear equation proposed by Seefeldt *et al.* (1995).

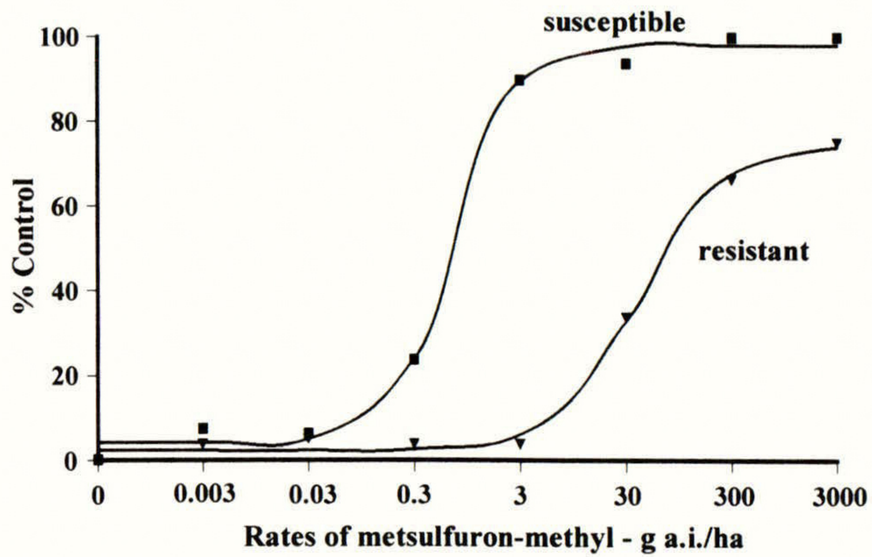
$$y = f(x) = C + \frac{D - C}{1 + (\frac{x}{GR_{50}})^b} = C + \frac{D - C}{1 + \exp(b(\log(x) - \log(GR_{50})))}$$

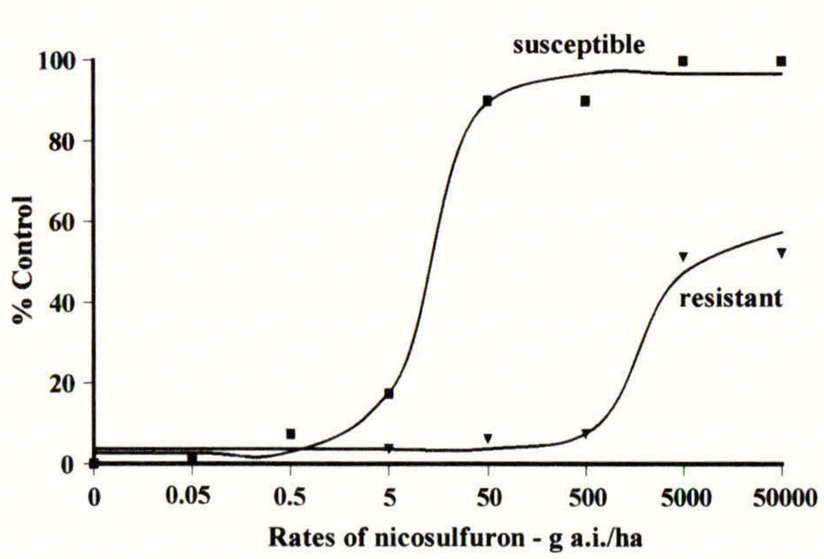
Where D = upper limit of the curve; C = lower limit of the curve; b = slope and GR50 = dose that corresponds to 50 % of the response. Graphics were constructed from these equations using log for the herbicide rates (independent variable).

RESULTS AND DISCUSSION

The parameters for the dose response curves for the resistant (R) and susceptible (S) biotypes are listed in Table 1. The GR_{50} values for the resistant biotype was statistically higher than the susceptible for all four herbicides studied. Therefore, R/S was significantly higher than one for all herbicides, showing that the R biotype of *B. pilosa* is resistant to ALS inhibitor herbicides tested (Table 2).







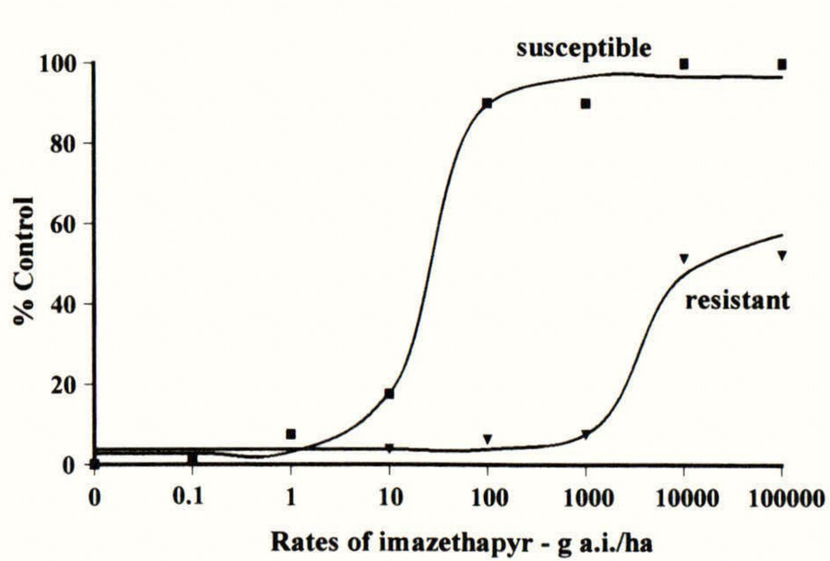


Figure 1. Dose response curves for resistant and susceptible *Bidens pilosa* to ALS inhibitors

The results also show that the R biotype has cross resistance to two classes of herbicides which inhibit ALS, i.e. sulfonylurea (chlorimuron-ethyl, nicosulfuron and metsulfuron) and imidazolinone (imazethapyr). The dose response curves are shown in Figure 1.

Table 1. Equation parameters of the dose response curves for the resistant and susceptible biotypes of *Bidens pilosa*

Herbicides	Biotypes	D	C	GR_{50}	b	R^2
Chlorimuron-ethyl	R	95.88	35.19	23.33	0.73	0.93
	S	98.12	-1.84	0.57	0.68	0.97
Nicosulfuron	R	96.28	42.25	43.46	1.74	0.94
	S	97.23	3.16	0.25	1.80	0.98
Metsulfuron methyl	R	97.73	25.46	13.22	1.12	0.93
	S	95.68	1.89	0.23	1.58	0.98
Imazethapyr	R	95.25	49.99	44.02	3.46	0.95
	S	95.74	5.62	0.77	1.35	0.97

Table 2. GR_{50} values of the resistant and susceptible biotypes with R/S values

Herbicides	Resistant (R)	Susceptible (S)	R/S
Chlorimuron-ethyl	466.6	11.4	40.92
Nicosulfuron	2173.0	12.5	173.84
Metsulfuron-methyl	39.66	0.69	57.47
Imazethapyr	4402.0	77.0	57.16

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The level of polyamines as an indicator of resistance or susceptibility of *Chenopodium album* to atrazine

J Giebel, S Stachecki, T Praczyk
Institute of Plant Protection, Miczurina 20, 60-318 Poznań, Poland

ABSTRACT

The level of free (PAs) and conjugated (CPAs) soluble polyamines in leaves of *Chenopodium album* was determined using the fluorimetric method for dansylated derivatives. The tests made at the 4-6 leaf growth stage showed that the concentration of PAs in susceptible (S) and resistant (R) biotypes ranged between 32-43 µg and 45-56 µg per 100 µl of cell sap, respectively. The analyses performed later, namely at the beginning of flowering showed that the difference in the level of polyamines between S and R biotypes was greater. In this case the amount of PAs in biotype S ranged between 15-34 µg and in biotype R between 51-73 µg per 100 µl. of cell sap. Therefore, the level of PAs cans act as an indicator for susceptibility or resistance of *C. album* biotypes to atrazine.

INTRODUCTION

During our studies on the effect of atrazine on *C. album* metabolism we paid attention particularly to the polyamines. These compounds can affect many processes in plant physiology, among others, changes in cell membrane fluidity, senescence and mitotic activity (Slocum et al., 1984). Unexpectedly we found that the level of polyamines in cell sap of the susceptible biotype of. *C. album* distinctly differed from that of the resistant one. Hence, it occurred to us that the level of polyamines in plants could be used as susceptibility/resistance indicator.

METHODS AND MATERIALS

We tested *C. album* plants of selected and homogeneous biotypes susceptible (S) and resistant (R) to atrazine, growing in greenhouse conditions (20/16 °C day/night respectively). Polyamines were detected in cell sap of the leaves sampled at the 4-6 leaf growth stage and then at the beginning of flowering. As we expected a large specimen variability, leaves from ten replicate plants of each biotype and growth stage were used.

For detection of PAs and CPAs, the method described by Smith & Davies (1987) was modified as shown on Figure 1. Soluble polyamines conjugated to small molecules (CPAs) were determined in cell sap after its hydrolysis with HCl (Torrigiani *et al.*, 1987). Hydrolysates contained initial PAs plus those liberated from various types of conjugated polyamines (CPAs). They were dansylated and determined as described in the scheme presented in Figure 1.

Dansyl-PAs were quantified by their relative fluorescence intensities (excitation at 365 nm, emission at 510 nm). using a fluorescence spectrophotometer (Perkin Elmer, model 650-10LC) and corrected using putrescin as an internal standard. Data are presented as an average of 3 replicates of each sample.

Cell sap + 0.2 N $HClO_4$ (1:1)

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Centrifugation (20 min, 48000g, 0^0 C). Supernatant contains PA and CPA

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To 100 µl supernatant add 200 µl sat. Na_2CO_3 + 400 µl 1% dansyl-Cl in aceton

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Sonification 3h – add 100 µl 10% proline – sonification 3 min

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Extraction of dans – PA with 500 µl of benzene

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Fluorimetric quantification and TLC of dans-PA

Figure 1. Scheme of analytical procedure for the detection of polyamines

Benzene extracts were applied to TLC Silicagel plates (DC-Alufolien Kieselgel 60) and then developed in ethyl acetate-cyclohexane (2:3 v/v). Spots were visualized by UV fluorescence.

RESULTS AND DISCUSSION

Our results show that the level of free polyamines (PAs) provide a good method for a chemotaxonomy of *C. album* biotypes (Table 1).

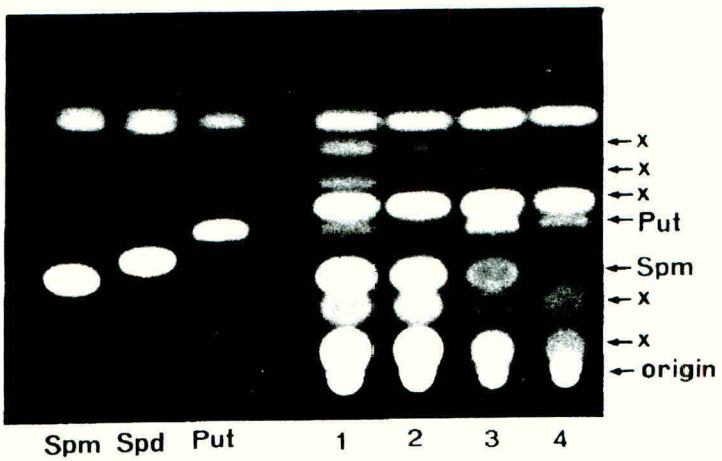

Figure 2. Free polyamines from cell sap of *Ch. album* leaves : biotype S (1) and R (2) at 4-6 leaf growth stage; biotype S (3) and R (4) at beginning of flowering.

Table 1. Soluble polyamines, free (PA) and conjugated (CPA), as µg of putrescin per 100 µl cell sap from of *C. album.*

	Susceptible biotype at 4-6 leaf growth stage				Resistant biotype at 4-6 leaf growth stage			
Sample	PA	PA+CPA	CPA	(PA+CPA):PA	PA	PA+CPA	CPA	(PA+CPA):PA
1	32	89	57	2.78	45	70	25	1.56
2	34	73	39	2.15	47	60	13	1.28
3	35	65	30	1.86	51	60	9	1.18
4	35	81	46	2.31	52	70	18	1.35
5	36	68	32	1.89	52	62	10	1.19
6	36	74	38	2.06	53	73	20	1.38
7	37	65	28	1.75	54	73	19	1.35
8	37	62	25	1.68	57	60	3	1.05
9	39	83	44	2.15	57	68	11	1.19
10	43	89	46	2.07	58	60	2	1.03
min.-max.	32-43	62-89	25-57	1.68-2.78	45-58	60-73	2-25	1.03-1.56
mean ± SD	36.4 ± 3.0	73.5 ± 10.1	38.5 ± 10.0	2.07 ± 0.32	52.5 ± 4.2	65.0 ± 5.7	12.0 ± 7.5	1.24 ± 0.16
	Susceptible biotype at beginning of flowering stage				Resistant biotype at beginning of flowering stage			
1	15	131	116	8.73	51	110	59	2.16
2	19	128	109	6.74	52	117	65	2.25
3	19	86	67	4.53	52	104	52	2.00
4	24	94	70	3.92	53	94	41	1.77
5	25	109	84	4.36	55	110	55	2.00
6	26	162	136	6.23	56	104	48	1.86
7	27	125	98	4.63	57	105	48	1.84
8	31	68	37	2.19	57	99	42	1.74
9	32	73	41	2.28	60	91	31	1.52
10	34	111	78	3.26	73	107	34	1.47
min.-max.	15-34	73-162	37-136	2.19-8.73	51-73	91-117	31-65	1.47-2.25
mean ± SD	25.5 ± 6.2	110.0 ± 29.1	81.0 ± 31.8	4.44 ± 2.05	55.5 ± 6.4	104.5 ± 7.8	48.0 ± 10.8	1.85 ± 0.25

Another approach could be the ratio of the sum of PA and CPA to PA. Here we can observe a large deviations in CPA levels. Probably, fluorimetric quantification of dansylated polyamines did not give such reliable results. We realize that the most sensitive method for detection of polyamines is their dansylation followed by spectrometry.

Dansyl derivatives are highly fluorescent and detectable in small amounts but, on the other hand, dansyl chloride used for derivatization is non-specific due to its high reactivity with amino groups of many compounds, namely some phenols and alcohol (Seiler & Wiechman, 1970).

TLC showed free putrescin and spermine in the cell sap of S and R biotype of *C. album* (Figure 2). However, five compounds occurring in detectable amounts labelled "x" in Figure 2, have not been identified so far.

CONCLUSIONS

The level of polyamines in cell sap of the susceptible biotype of. *C. album* distinctly differed from that of the resistant one. Therefore, the level of free polyamines can act as an indicator for susceptibility or resistance of *C. album* to atrazine.

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Mechanism of isoproturon resistance : the metabolism of isoproturon in susceptible and resistant biotypes of *Phalaris minor*

G Kulshrestha, S B Singh
Division of Agricultural Chemicals, IARI, New Delhi - 110012 India

N T Yaduraju
Division of Agronomy, IARI, New Delhi - 110012 India

ABSTRACT

Possible mechanisms of resistance to the photosynthetic inhibiting herbicide isoproturon were examined in a resistant biotype of *Phalaris minor* from India. Experiments were conducted under controlled and field conditions to evaluate metabolic fate of isoproturon. The resistant biotype tolerated a three times higher dose than the susceptible biotype. In a field experiment, isoproturon was completely degraded in 8 days in the resistant biotype and wheat, but persisted up to 18 days in the susceptible biotype. The toxic monodesmethyl and didesmethyl analogues were found in the susceptible biotype for up to 18 days. In the resistant biotype and wheat, two additional nontoxic metabolites, *p*-isopropyl aniline and a hydroxy derivative, were detected from 8 days onward confirming that metabolism of isoproturon in resistant *P. minor* was similar to that in wheat.

INTRODUCTION

Phalaris minor is a common grass weed mostly associated with winter cereals including wheat in India (Malik & Singh, 1993) . It is an intensively competitive weed in cereal crops and reduces wheat yield. *P. minor* has been adequately controlled in wheat by substituted urea herbicides including methabenthiazuron, metoxuron and isoproturon.

Almost fifteen years after the introduction of the phenylurea herbicides, *P. minor* biotypes resistant to isoproturon were identified (Malik & Singh, 1995) in certain North-West regions of India. Weeds with differing resistance mechanisms (Yaduraju & Ahuja, 1995; Singh *et al.*, 1998) have been identified. Subsequently, over ten populations of herbicide resistant *P. minor* have been reported from Haryana and Punjab in India (Walia *et al.*, 1997). Some cereals including wheat are tolerant to isoproturon enabling this herbicide to selectively control weeds in this crop (Kulshrestha, 1982). This is primarily due to rapid metabolism to non-toxic compounds. Tolerant crop plants detoxify phenylurea herbicides by two major reactions: N-demethylation with a hydroxylated intermediate and an aryl ring hydroxylation both of which may be followed by glucose conjugation. The overall rate of chlortoluron degradation in the weed *Alopecurus myosuroides* is slower than in wheat which explains why this weed can be selectively controlled in wheat crop (Ryans & Owen, 1982). The

objective of this investigation was to ascertain the mechanism of resistance to isoproturon in a biotype of *Phalaris minor*.

MATERIALS AND METHODS

Source of seeds, isoproturon and metabolites

Seeds of susceptible (S) *P. minor* were collected from winter wheat fields of Delhi in 1995 and 1996. The resistant (R) biotype was collected from a field in Haryana where pronounced resistance to isoproturon was first detected in 1985 (Malik & Singh, 1993). Wheat was grown in this field for over 25 years and had received applications of isoproturon every year from 1979 to 1997. Analytical grade isoproturon and metabolites were supplied by Gharda Chemical Ltd. and the commercial formulation of isoproturon (Arelon, 50 WP) was supplied by Hoechst India Ltd. The identity of the metabolites was established earlier (Kulshrestha & Mukerjee, 1986).

Metabolism of isoproturon

In a laboratory study seeds were germinated on cotton wool in a petridish until coleoptile emergence and then transplanted in to 3 cm diameter plastic pots containing alluvial soil and kept in a phytotron under controlled environmental conditions: 20/16°C day/night temperatures, 16 h photo period. At the three leaf stage, soil was washed from the roots and plants were transferred to foil covered beakers containing 10 ml of isoproturon saturated (520 µg/ml) buffer solution (pH 7). After 48 h the roots of individual plants were rinsed with distilled water, excised to divide into root and shoot. Plant parts were macerated in a mortar and pestle with anhydrous sodium sulphate in 10 ml methanol.

In the field study, seeds of the *P. minor* biotypes were sown in the field in rows alternate with wheat plants. Seedlings at the 3-leaf stage were sprayed with isoproturon at 0.75 kg a.i./ha using a knap-sack sprayer calibrated to deliver 250 litres/ha. After 0, 1, 2, 4, 8 and 18 days representative plants of each biotype were removed together with roots, brought to the laboratory, divided into roots and shoots, washed in 20 ml water and extracted. The plants were ground in a mortar and pestle and extracted with 10 ml extraction solution (80% methanol, v/v) at 25°C, rinsed with 5 ml extraction solution and centrifuged for 20 min at 12,000 rpm. The pellet was re-extracted twice with 5 ml of extraction solution and recentrifuged and supernatants were combined and solvent evaporated in a rotary evaporator. The aqueous phase was partitioned with hexane (10 ml) and hexane was discarded. The aqueous phase was finally partitioned with dichloromethane (25+10+10 ml), passed through anhydrous sodium sulphate and evaporated in *vacuo* to dryness. The residue was resuspended in 500 µl acetonitrile. Parent herbicide and metabolites were quantitatively analysed by HPLC (HP LC 1100, equipped with C-18 reverse-phase ODs column, and photodiode-array detector at 254 nm). Chromatographic conditions were modified from Kulshrestha & Khazanchi (1991) and consisted of a mobile phase of acetenitrile: water (60:40). Each experiment consisted of three replicates containing 10 plants of each biotype for each time point and was repeated twice. Data were pooled and the quantity of herbicide

and metabolites were expressed as µg/g plant sample. Isoproturon and authentic metabolite standards were chromatographed separately and simultaneously using the conditions described earlier.

RESULTS

Metabolism of isoproturon

The R biotypes rapidly metabolised isoproturon (Figure 1). Plants harvested 6 h after treatment with isoproturon contained only 9 µg/g plant of isoproturon, and this declined over the next 48 h, to 6.6 µg/g plant and after 18 days the isoproturon was completely metabolised. In the S biotype 6 h following herbicide treatment, 43 µg/g of isoproturon was present and decreased to 10 µg/g over the next 48 h. In wheat plants, 17 µg/g of isoproturon remained after 6 h and decreased to 10 µg/g in the following 48 h. Isoproturon was not detected after eight days. A similar pattern of isoproturon absorption and degradation was observed in the roots of the two biotypes of *P. minor*. Isoproturon persisted in roots up to 18 days in the S biotype compared to 4 days and 2 days in the R biotype and wheat (Figure 1) respectively.

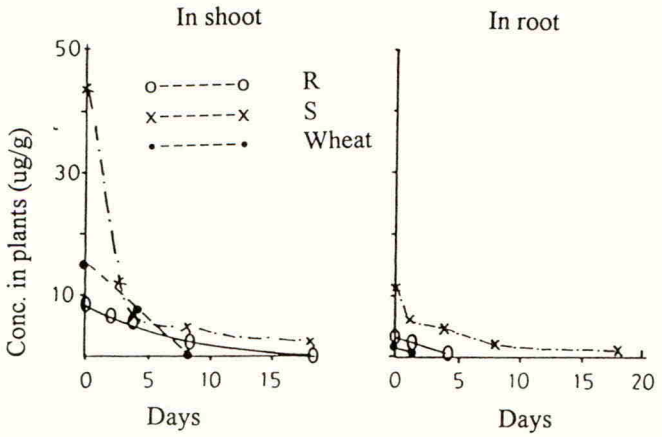

Figure 1. Isoproturon remaining in shoot and root of wheat, R & S biotypes of *P. minor* over 18 days following field application at 0.75 kg a.i./ha.

Metabolite I tentatively identified by cochromatography as the partially toxic monodemethylated metabolite (Figure 2) was initially greater in the S biotype, 0.113 µg/g plant compared to 0.02 and 0.015 µg/g plant in the R biotype and wheat, respectively (Figure 3).

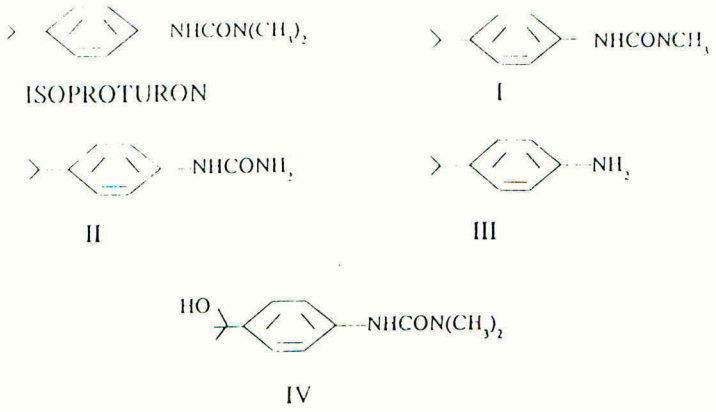

Figure 2. Chemical structures of isoproturon and metabolites.

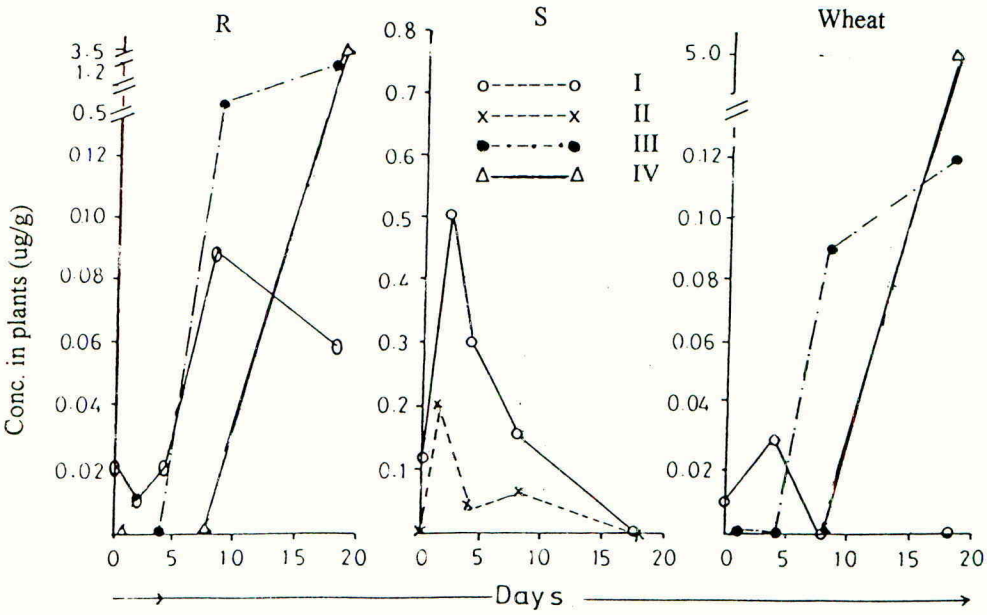

Figure 3. Concentration of metabolites recovered from wheat, and R and S biotypes of *P. minor* over 18 days following application of isoproturon at 0.75 kg a.i./ha.

Metabolite I decreased over 48 h but increased thereafter in the R biotype but in the S biotype its concentration increased over 48 h and then decreased for the remainder of the experiment and it was much more abundant. Metabolite II tentatively identified by

cochromatography as the partially toxic didesmethylated metabolite, was found in the S biotype 48 h after treatment, decreased thereafter and disappeared in 18 days (Figure 3). In wheat 17 µg/g isoproturon was recovered after 6 h which decreased to 8.7 µg/g after 4 days and was absent after 8 days. The Monodesmethyl metabolite was found in low amounts up to 8 days but didesmethylated metabolite was absent. Rather high amounts of nontoxic metabolite III formed from day 8 onwards in the R biotype and wheat, but was absent in the S biotype. Metabolite IV, tentatively identified by cochromatography as nontoxic 4-(1-hydroxy-1-methyl ethyl) phenyl-N',N'-dimethyl urea, was found in the R biotype and wheat within 48 h of treatment. The proportion of all metabolites increased in the R biotype as isoproturon was degraded. Similar non-toxic metabolites III and IV were found in both wheat and the R biotype under phytotron conditions (Figure 4).

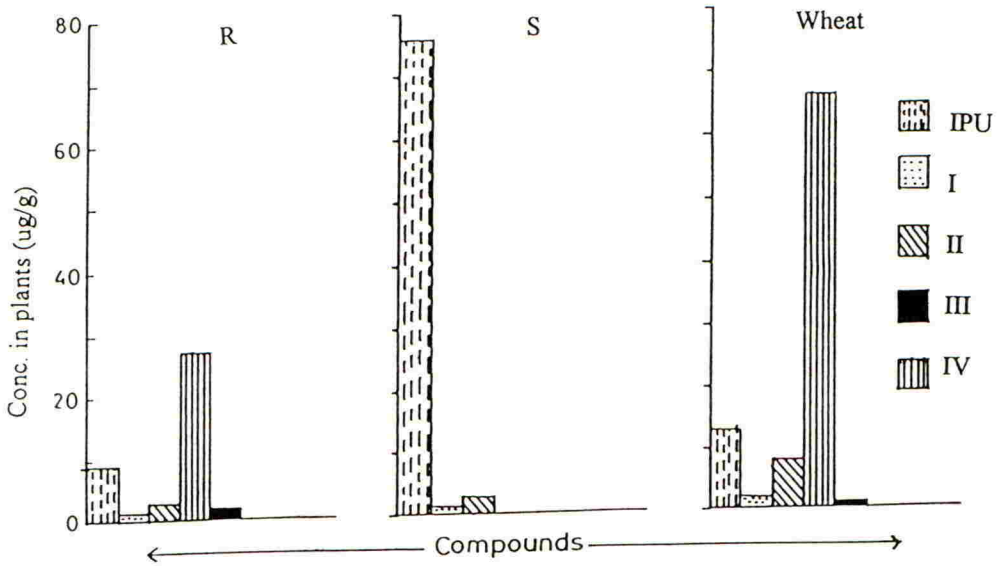

Figure 4. Concentration of isoproturon and metabolites formed in R and S biotypes of *P. minor* and wheat after 48 h exposure to isoproturon saturated buffer (pH 7) under phytotron conditions.

DISCUSSION

The Phalaris minor biotype which is resistant to isoproturon exhibits cross resistance to most of the substituted phenylurea herbicides and some other herbicides such as diclofop-methyl, (Singh *et al.*, 1999). It is evident from the data in Figure 1 that resistance in the R biotype is due to enhanced isoproturon metabolism. The relative amount of isoproturon metabolised by this biotype corresponds to the level of herbicide resistance in whole plants sprayed with isoproturon or treated with isoproturon hydroponically. Figures 3 and 4 show rapid metabolism of isoproturon in the R compared to S biotype which confirms and extends previously reported data (Singh *et al.*, 1998).

The R and S biotypes and wheat produce metabolites I, II, III and IV (Figure 2). The major difference between R and S biotypes is complete transformation of isoproturon in eight days in the R biotype with increased formation of metabolites I, III and IV. The major pathway of isoproturon degradation in the R biotype appears to be aryl alkyl hydroxylation leading to the nontoxic hydroxy isopropylphenyl metabolite (IV). Interestingly the pattern of isoproturon degradation in the R biotypes is similar to wheat where too isoproturon was completely dissipated from plants in eight days with increased formation of metabolites III and IV. The cytochrome P450 monooxygenase enzymes are known to detoxify chlortoluron and isoproturon in wheat and the R biotype of *P. minor* has probably acquired higher activities of these enzymes as the metabolites formed in wheat and resistant *P. minor* biotypes are similar.

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An investigation of glutathione *S*-transferase activity in *Alopecurus myosuroides* Huds. (black-grass) in the field

L J Milner, J P H Reade and A H Cobb

Crop and Environment Research Centre, School of Agriculture, Harper Adams University College, Newport, Shropshire, TF10 8NB, UK

ABSTRACT

Herbicide resistance is being investigated in *Alopecurus myosuroides* Huds. (black-grass) populations in field and glasshouse experiments with respect to glutathione *S*-transferase (GST) activity. GSTs are a group of enzymes that have been implicated in many roles in plant metabolism, including herbicide detoxification and resistance. Previous experiments have indicated that the resistant black-grass biotype Peldon contains approximately double the GST activity of susceptible biotypes and have suggested that there is a correlation between GST activity and herbicide resistance in other biotypes. The relationship between GST activity and black-grass growth and development has been investigated. Studies have revealed that as untreated plants in the field mature, there is an accompanying natural elevation of GST activity with environmental changes from winter to spring. It is speculated that this endogenous change in enzyme activity with plant development in the field contributes to reduced efficacy of some graminicides applied in the spring.

INTRODUCTION

Black-grass (*Alopecurus myosuroides* Huds.) is a common arable grass weed which presents major problems in autumn grown cereal crops in the UK, through reduction of both crop quality and yield (Moss, 1987). Since the initial detection of herbicide resistance in this species in the early 1980s (Moss & Cussans, 1985), over 750 farms in 30 counties throughout England have been confirmed as having resistant black-grass populations (Moss, 1997). Many of these populations also exhibit cross-resistance to a range of herbicides from differing chemical groups with differing modes of action (Willis *et al.*, 1997). There is clear evidence to suggest that more than one resistance mechanism exists in black-grass. Recent research suggests that resistance is due in part to enhanced metabolism involving both cytochrome P450 monooxygenases and glutathione S-transferases (Cummins *et al.*, 1997; Hall *et al.*, 1997; Reade *et al.*, 1997). Reade *et al.*, (1997) have indicated that the resistant black-grass population Peldon contains approximately double the GST activity of susceptible biotypes and have suggested that there is a correlation between GST activity and herbicide resistance. GST's are a group of enzymes, which catalyse conjugation of the tripeptide glutathione to a variety of hydrophobic and electrophilic substrates. They are involved in cellular metabolism and have been intensively studied with regard to detoxification of agrochemicals, especially herbicides, in plants as reviewed by Marrs (1996). In this study, herbicide resistance has been studied in black-grass populations within the field with respect to glutathione *S*-transferase activity.

Individual leaves sampled at each growth stage from both sites were analysed. The results indicate that mean endogenous GST activity within individual leaves significantly increased ($P<0.05$) as plants matured and developed from juvenility at GS13 through to relative maturity at GS57 and GS31 for Sites 1 and 2 respectively, as shown in Figure 1.

Further statistical analysis through linear regression indicated that at each sampling growth stage, there was no significant difference ($P>0.05$) in mean GST activity (µmol CDNB min^{-1} g^{-1} fwt) between the individual leaves of each plant sampled, suggesting that data from the single leaves of one plant can be averaged to indicate a whole plant response as shown in Figure 2.

Protein content

Mean soluble protein values (mg g^{-1} fwt) for individual leaves also showed significant increases ($P<0.05$) at different developmental stages at both sites (data not shown).

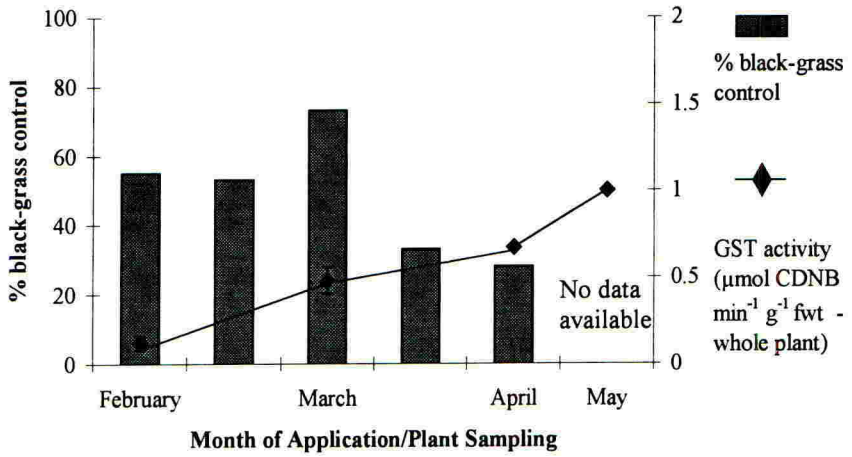

Figure 2. Effect of application timing on the control of black-grass by fenoxaprop-p-ethyl (Novartis). Additional data on mean GST activity (µmol CDNB min^{-1} g^{-1} fwt – whole plant) is overlaid for comparison. GST values are means SE± values, where n = 5 whole plants. Error bars are included where they exceed the symbol size.

DISCUSSION

Black-grass resistance to herbicides is a continuing problem for UK farmers. There are many commercially available chemicals together with guidelines for its control, but the problem for some farmers has become progressively worse each year. Research to investigate the underlying reasons for reduced herbicide efficacy and guidelines to avoid it are therefore important when striving for maximum yields and profits.

These findings indicate that there is a natural elevation of endogenous GST activity with the development of untreated black-grass plants from February to May 1999. However, the functions of GSTs in cellular metabolism and their activity towards endogenous substrates remains largely unexplored (Edwards, 1995). This progressive increase in enzyme activity may simply reflect the development of black-grass leaves and whole plants i.e. juvenility to senescence as shown by Badiani *et al.*, (1996) in wheat leaves. There is no evidence to suggest that these natural elevations in activity are due to stress induced by abiotic, biotic or environmental/climatic factors.

The effects of black-grass on cereal yields and the relative earliness in the season with which it becomes established, dictates that it should be controlled in the autumn if maximum yield potential is to be realised. Incidence of decreased herbicide efficacy due to black-grass plants maturing, effects of resistance and environmental factors is well-documented (Blair *et al.*, 1996). The role of GSTs in determining herbicide tolerance and thus selectivity through detoxification within crops and weeds is well established. The increase in innate GST activity shown here may offer an explanation of the overall trend of reduced herbicide efficacy observed from February onwards in the field (Figure 2). The results from this study, together with observations from Novartis, indicate that the timing of herbicide application is critical, especially where resistant black-grass populations are suspected. We have also established that more tolerant plants contain higher GST activity (Reade and Cobb, 1999). Indeed, the resistant Peldon biotype exhibits double the GST enzyme activity of susceptible biotypes, due to constitutive GST enzyme activity rather than that induced by herbicide treatment, as indicated by Reade *et al.*, (1997). In addition, as proposed by Cummins *et al.*, (1997), it is possible that GST subunits accumulate during specific phases of development or environmental cues e.g. the transition of winter to spring, as indicated in Figure 2.

Many commercially available herbicides are proposed for black-grass control up to GS39 (flag leaf ligule visible). Our findings indicate that the role of GSTs in terms of herbicide selectivity could be dependent on the developmental stage of the weed. It may be that herbicide recommendations need to be revised in order to avoid a decrease in efficacy and thus potentially greater yield losses. The current observations point towards innate GST activity within black-grass plants being partly responsible for reduced herbicide efficacy in the field. We therefore speculate that changes in GST activity normally occur during plant growth and that our observations lend further weight to the suggestion that the development of resistance in black-grass is in part due to evolution and elevation of GST activity. It may be that this knowledge could be utilised in predicting how populations of black-grass are treated in the future with respect to identifying resistance and which herbicides are likely to be ineffective with regard to successful control.

ACKNOWLEDGEMENTS

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The occurrence of herbicide-resistant grass-weeds in the United Kingdom and a new system for designating resistance in screening assays

S R Moss
IACR-Rothamsted, Harpenden, Herts, AL5 2JQ, UK

J H Clarke, A M Blair
ADAS Boxworth, Boxworth, Cambridge, CB3 8NN, UK

T N Culley
Zeneca Crop Protection, Fernhurst, Haslemere, Surrey, GU27 3JE, UK

M A Read
AgrEvo UK Ltd, East Winch Hall, King's Lynn, Norfolk, PE32 1HN, UK

P J Ryan
Novartis Crop Protection UK Ltd, Whittlesford, Cambridge, CB2 4QT, UK

M Turner
Oxford Plant Sciences Ltd, Bainton Road, Bucknell, Bicester, Oxford, OX6 9LT, UK

ABSTRACT

Several organisations conduct screening tests for resistance in the UK. A compilation exercise was undertaken to determine how many individual farms had been identified as containing resistant grass-weeds. Resistant black-grass (*Alopecurus myosuroides*) has been found on 746 farms in 30 counties, resistant wild-oats (*Avena* spp.) on 65 farms in 19 counties and resistant Italian rye-grass (*Lolium multiflorum*) on 25 farms in 11 counties of England. No resistant grass-weeds have so far been detected in Wales, Scotland or Northern Ireland. These results show that resistance occurs over a very wide geographical area.

At present, different organisations do not interpret screening results in the same way in terms of designating degree of resistance. A new system is proposed, which while based on the * rating system developed by IACR-Rothamsted/ADAS, requires the use of only a single susceptible standard. This new system is appropriate for wild-oats and Italian rye-grass as well as black-grass, and is applicable to a wider range of herbicides. This new system assigns samples to four categories, RRR, RR, R? or S (susceptible).

INTRODUCTION

Herbicide-resistant black-grass (*Alopecurus myosuroides*) was first detected in England in 1982, resistant Italian rye-grass (*Lolium multiflorum*) in 1990 and resistant wild-oats (*Avena* spp.) in 1993. In recent years there has been increasing interest in resistance, especially to aryloxyphenoxypropionate ("fop") and cyclohexanedione ("dim") herbicides, as new herbicides from these two groups have become available for grass-weed control in cereals. Fenoxaprop-ethyl was launched onto the UK market in 1990, tralkoxydim in 1993 and

clodinafop-propargyl in 1995. Concerns about resistance resulted in companies initiating their own in-house testing for resistance in addition to testing conducted by research organisations. The location of farms with resistance was kept confidential by each testing organisation/company so it was likely that there was some duplication of testing. Without any cross-referencing system, there was no way of knowing how much double counting was occurring. The BCPC Weeds Sub-Committee proposed that an attempt be made to collate the results of all tests in order to obtain a more precise figure for the number of farms with confirmed resistance. In addition, as different organisations/companies do not interpret the results of resistance screening assays in exactly the same way, it was decided to develop a standardised method which all centres should then adopt.

OCCURRENCE OF HERBICIDE-RESISTANT GRASS-WEEDS

All companies (AgrEvo, Novartis, Zeneca) and organisations (ADAS, Oxford Plant Sciences, IACR-Rothamsted) which conduct resistance screening tests were invited to submit results, in confidence, to S R Moss (IACR-Rothamsted), who collated the information on a county basis. This was undertaken in 1996 for black-grass and in 1999 for wild-oats and Italian rye-grass.

It is important to note that:

- This is in no way a random survey. Samples were mainly collected following a report of inadequate control by a herbicide. ADAS did collect black-grass samples on a random basis between 1988 and 1991, but these now account for less than 5% of the total resistant samples. No random surveys of resistant wild-oats or Italian rye-grass have ever been undertaken in the UK.

- The testing method used was a glasshouse pot assay, similar to that described by Clarke *et al.*, (1994). The herbicides used on black-grass were chlorotoluron, isoproturon, fenoxaprop-ethyl or fenoxaprop-P-ethyl and clodinafop-propargyl. Very few populations were tested for resistance to all four herbicides. The standard herbicides used to determine resistance in the other species were fenoxaprop-P-ethyl on wild-oats and diclofop-methyl on Italian rye-grass.

- The results of glasshouse pot tests can be open to different interpretation. This is more of a problem with resistance to substituted-urea herbicides than to "fops" where resistance tends to be more clear cut (Moss *et al.*, 1998). The results presented here are based on the designation of resistance by each individual company or organisation.

- For reasons of confidentiality, no national list of addresses of farms with resistant black-grass has been produced. Farms were identified by codes and distribution of resistance has only been defined down to the county level.

Black-grass

- Resistance to chlorotoluron or isoproturon (substituted-urea herbicides) was detected on 127 farms.
- Resistance to fenoxaprop or clodinafop ("fop" herbicides) was detected on 691 farms.
- Some farms were in both groups, so the number of farms with confirmed resistance to one or more herbicides was **746** distributed over **30 counties** in England (Figure 1).
- The counties with the greatest recorded number of farms with confirmed resistance were: Lincolnshire – 117; Essex – 103; Oxfordshire – 92; Cambridgeshire – 75; Suffolk – 58.
- The total number of farms where resistance was detected (746) represents about 3.7% of the estimated 20,000 cereal farms in the UK with a black-grass problem (out of a total of about 50,000 farms growing cereals). However, the majority of farms (>90%) have not had any sample tested for resistance. This figure (3.7%) should not be used to indicate the proportion of farms with resistant black-grass in the UK, only as a **minimum** figure for proportion of farms affected.
- No resistant black-grass has so far been detected in Wales, Scotland or Northern Ireland.

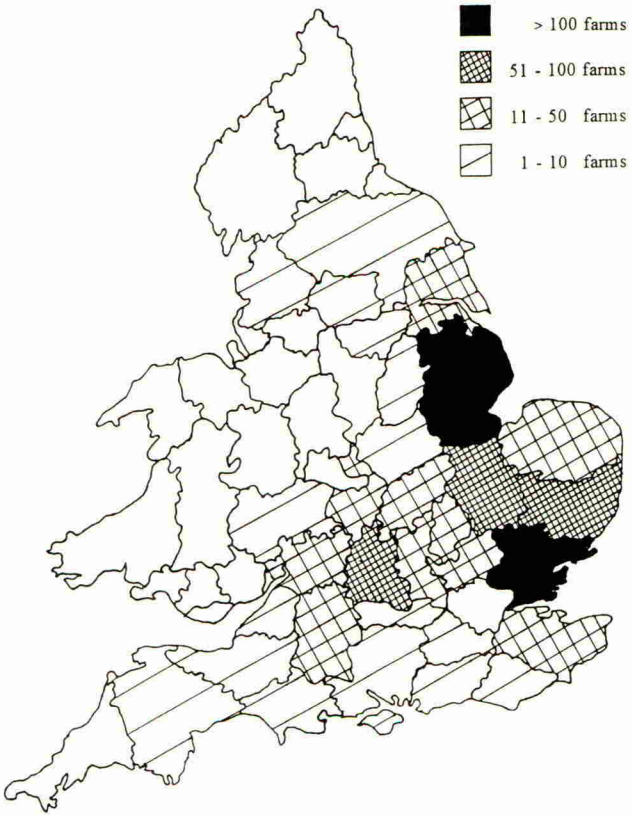


Figure 1. The distribution of farms in England, by county, where black-grass resistant to at least one herbicide (chlorotoluron, isoproturon, fenoxaprop or clodinafop), had been detected by 1995

Wild-oats

- Resistance to fenoxaprop-P-ethyl was detected on **65 farms** distributed over **19 counties** of England (Table 1).
- The counties with the greatest recorded number of farms with confirmed resistance were: Essex - 17; Lincolnshire – 7; Norfolk – 7; Cambridgeshire – 6; Somerset – 6.
- Resistant wild-oats have not been detected in Wales, Scotland or Northern Ireland.
- Although fenoxaprop was used as the standard herbicide for screening for resistance, cross-resistance to herbicides with the same, and different mode of action, often occurs.

Italian rye-grass

- Resistance to diclofop-methyl was detected on **30 farms** distributed over **12 counties** of England (Table 1).
- The counties with the greatest recorded number of farms with confirmed resistance were: Essex – 6; Norfolk – 6.
- Resistant Italian rye-grass has not been detected in Wales, Scotland or Northern Ireland.
- Although diclofop-methyl was used as the standard herbicide for screening for resistance, cross-resistance to herbicides with the same, and different mode of action, often occurs.

Table 1. The distribution of farms in England, by county, where wild-oats resistant to fenoxaprop-P-ethyl and Italian rye-grass resistant to diclofop-methyl had been detected by 1999.

County	Wild-oats Number of farms	Italian rye-grass Number of farms
Bedfordshire	1	0
Berkshire	1	0
Cambridgeshire	6	2
Devon	1	0
Dorset	1	1
Essex	17	6
Kent	2	2
Gloucestershire	2	0
Humberside	1	0
Leicestershire	1	2
Lincolnshire	7	3
Norfolk	7	6
Northamptonshire	1	1
Nottinghamshire	2	1
Oxfordshire	1	1
Somerset	6	0
Suffolk	3	0
Warwickshire	3	0
Wiltshire	2	3
Yorkshire	0	2
TOTAL	65 farms in 19 counties	30 farms in 12 counties

A NEW SYSTEM FOR DESIGNATING RESISTANCE IN SCREENING ASSAYS

Background

Most centres testing for resistant black-grass use the standard glasshouse pot test in which seeds are sown in pots and plants sprayed at the 2-3 leaf stage with herbicides. Some centres make a visual assessment of reduction in foliage or a vigour score while others do a more objective assessment of foliage weight. Most centres in the UK use fenoxaprop as a standard, but may also include additional herbicides such as chlorotoluron or isoproturon. A key conclusion of a resistance "ring test" was that, regardless of how screening assays are conducted, the basis on which resistance is assigned should be stated (Moss *et al.*, 1998).

At present different centres do not interpret the results from screening assays in the same way in terms of designating degree of resistance. Some use the * rating system while others use a simpler categorisation based on visual scores. The original * rating system (Clarke & Moss, 1989), required the use of three standard black-grass populations which were used in every test. This system was only really applicable to chlorotoluron. A revised system (Clarke *et al.*, 1994), required the use of only two standards (Rothamsted, Peldon) and this was suitable for chlorotoluron and fenoxaprop. Faringdon (the third standard) had to be dropped as seed supplies were limited and it was relatively more resistant to fenoxaprop than chlorotoluron.

Increasingly it has been recognised that a system is required which is:

- applicable to other species (e.g. wild-oats, Italian rye-grass) as well as black-grass.
- applicable to a wider range of herbicides.
- less dependent on the provision of resistant standards which may only be applicable to a narrow range of herbicides and which may be difficult to maintain long term.

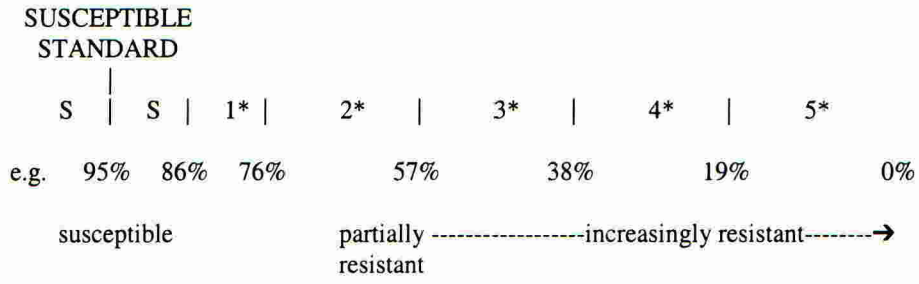
The new "R" system

The UK Weed Resistance Action Group (WRAG) has proposed that the following system should be used by all UK centres screening black-grass for resistance in single dose assays, so that this aspect of interpretation is standardised. The system is summarised below and is appropriate for wild-oats and Italian rye-grass as well. It may also be appropriate for other resistant weeds and for Petri-dish, as well as pot assays.

The latest version of the * rating system requires the inclusion of just a single standard reference population for each species in every test – a susceptible standard. The latest version retains the advantages of the previous system in terms of accommodating a continuum of responses, allows for a slight reduction in number of resistance categories and utilises the same susceptible standard for all herbicides.

A vital prerequisite is that control (e.g. % reduction in foliage weight) of the susceptible standard should be over 80%. The % reduction values between the susceptible standard and zero are separated into five equal categories (see diagram). One of these categories, at the susceptible end of the range, is subdivided about its mid point into two smaller categories, S and 1*. Any populations more sensitive than the susceptible standard are also termed susceptible. It is important to stress that the determination of the different categories is made using the % reduction value obtained for the standard susceptible population in each

individual test. The actual values delineating the categories will differ between tests. Thus if the susceptible % reduction value was 95%, each category would be 19% (i.e. 95% ÷ 5 = 19%). Thus <19% = 5*; 19% to 38% = 4*; 38% to 57% = 3*; 57% to 76% = 2*; 76% to 85.5% = 1*; 85.5% to 95% (and over) = S (susceptible).



The higher the * rating the greater the degree of resistance. The results only relate to the actual sample and herbicide tested. In practice, the six categories calculated above are more than are needed for screening purposes, so the following four-category system is suggested with appropriate descriptions. **It is recommended that testers designate samples RRR/RR/R? or S, instead of giving * ratings:**

5*/4* = **RRR Resistance confirmed, highly likely to reduce herbicide performance**
3*/2* = **RR Resistance confirmed, probably reducing herbicide performance**
1* = **R? Early indications that resistance may be developing, possibly reducing herbicide performance**
S = **S Susceptible.**

This system incorporates a risk element in that the higher the degree of resistance the greater the risk of herbicide failure, and gives farmers an indication of the implications.

ACKNOWLEDGEMENTS

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Rapid tests for herbicide resistance in black-grass based on elevated glutathione *S*-transferase activity and abundance

J P H Reade, J L Belfield, A H Cobb
Crop and Environment Research Centre, School of Agriculture, Harper Adams University College, Newport, Shopshire, TF10 8NB, UK

ABSTRACT

Black-grass (*Alopecurus myosuroides*) is a major problem weed in winter cereal crops in the UK. Control is hindered by the presence of black-grass biotypes resistant to herbicides. Early detection of these biotypes would allow alternative crop protection measures to be employed. Data are presented demonstrating raised glutathione *S*-transferase (GST) activities in black-grass plants that have survived various herbicide treatments in field trials. In addition, the detection of raised GST protein abundance in these plants using enzyme-linked immunosorbent assay (ELISA) technology is described. The ELISA utilises antisera raised against a GST subunit from the resistant black-grass biotype Peldon, purified in this laboratory. Changes in GST activity from February to May 1999 are also presented. Detection of GST activity (by colorimetry) and GST abundance (by ELISA) is discussed in relation to the development of a quick field-test for herbicide-resistant biotypes that will give accurate results before application of post-emergent herbicides.

INTRODUCTION

Black-grass (*Alopecurus myosuroides* Huds.) is a major problem weed in winter cereals in the UK. Satisfactory control, by both chemical and cultural means, is important to maintain both crop quality and yield. Although selective graminicides have proved effective in the control of black-grass in cereals, the increase in populations demonstrating herbicide resistance to these means satisfactory control is not always achieved. Herbicide resistance in black-grass in the UK was first reported against chlortoluron (CTU) in the early 1980s (Moss & Cussans, 1985) and by 1996 more than 750 UK farms had black-grass demonstrating some form of herbicide resistance. This resistance may be to a single herbicide, although cross-resistance (due to a single resistance mechanism) and multiple resistance (due to the presence of more than one resistance mechanism) are increasingly encountered (Heap, 1997).

Previous research in this laboratory has demonstrated that the resistant black-grass biotype Peldon contains approximately double the activity of the enzyme glutathione *S*-transferase (GST) (Sharples *et al.*, 1995). Further study with this biotype has revealed that raised activity may be due, at least in part, to the presence of a GST subunit not detected in susceptible plants (Reade & Cobb, 1999). This 30 kDa subunit was present in the Peldon biotype along with a 27.5 kDa subunit also found in susceptible biotypes. GSTs have been widely reported to metabolise herbicides (for a review, see Marrs, 1996). It is possible that they can be utilised as a marker of the

herbicide susceptibility of a particular black-grass biotype, and that raised GST activity or abundance might form the basis of a simple test for herbicide resistance.

A test to determine the extent of herbicide resistance in black-grass populations would aid in its control. Present tests for resistance utilise seeds collected from black-grass prior to crop harvest (Moss, 1999). Hence, seed is returned to the soil and resistance control measures can only be carried out in the following season. Alternative tests involve glasshouse spraying of black-grass plants that have survived herbicide treatment in the field, and can be costly and time consuming. Thus, existing testing can only be accomplished with seed or transplanted plants over a minimum of 6 weeks. Our research is directed towards the development of a quick resistance test that can be carried out prior to post-emergent herbicide application, so allowing alternative measures to be adopted, where necessary, during the same season as detection. It is envisaged that this test will give results only hours after collection of plants.

In this study, both GST activity and the presence of anti-GST ELISA-detectable polypeptides were assessed in black-grass plants that had survived various herbicide treatments during field trials. These results are compared to those obtained for untreated plots, and the possibility of a resistance test, to be carried out prior to post-emergent herbicide treatment, is discussed.

MATERIALS AND METHODS

Plant material

Black-grass plants were collected from thee UK field sites (courtesy of Novartis Crop Protection UK Ltd.). Sites 1 and 3 had received various treatments on separate plots (Table 1), and were sampled once. Site 2 received no herbicide treatment this season (1998/99), but had been treated with a variety of herbicides on separate plots for the previous 6 years (Table 1). This site was sampled four times between February and May 1999.

All above ground biomass was harvested from ten plants, where possible, from each plot. Tissue was frozen on dry ice for transportation to the laboratory.

Protein extraction and GST assay

Proteins were extracted and GST activities determined using the method of Reade *et al.* (1997). Each plant was extracted in 100 mM phosphate buffer (pH 7.0), desalted and stored at –80 °C. Dilutions for ELISA detection were carried out using the extraction buffer. Protein determinations were carried out using an adaptation of the method of Bradford (1976).

ELISA detection of GST polypeptides in extracts

Antisera were raised against a 30 kDa GST subunit purified from the herbicide-resistant biotype Peldon (Reade & Cobb, 1999). These antisera were used to detect

polypeptides in extracts from site 2 (Feb 1999). Immunodetection was carried out on 96 well plastic plates. Extracts were diluted to a protein concentration of 1 μg/50 μl and 1 μg was loaded per well overnight at 4 °C. Wells were then blocked for 3 h using 3 % (w/v) milk powder (Marvel Original, Premier Beverages, Stafford, UK) at 37 °C. Antiserum raised against the 30 kDa GST subunit purified from the black-grass biotype Peldon (Reade & Cobb, 1999) was incubated with the immobilised protein overnight at 4 °C. Following a wash step, the wells were incubated with goat anti-mouse alkaline phosphatase conjugate (Dako Ltd., Cambridgeshire, UK) for 2 h at room temperature with shaking. Following a second wash step, the antibody conjugate was detected using 1 mg/ml *p*-nitrophenyl phosphate. Colour development was stopped using 3 M NaOH. Absorbance was measured immediately at 405 nm. At least three replicates were carried out for each sample.

Table 1. Herbicide treatments at the thee field sites. Sites 1 and 3 received treatments on 4th November and 16th December 1998 respectively, when black-grass was at GS 11-13. Site 2 received no treatment during the season of the study, but received the treatments described below for 6 years preceding the study.

Plot / Site	1	2	3
1	IPU (2500 g a.i. ha) as Auger (5 litres/ha)	Diclofop (900 g a.i./ha) as Illoxan-European (2.4 litres/ha)	--------
2	IPU (2500 g a.i. ha) as Auger (5 litres/ha)	Clodinafop (30 g a.i./ha) as Topik (0.125 litres/ha)	Fenoxaprop-P (69 g a.i./ha) as Cheetah S (1.25 litres/ha)
3	IPU (2500 g a.i. ha) as Auger (5 litres/ha)	Diclofop (900 g a.i./ha) as Illoxan-European (2.4 litres/ha)	--------

RESULTS

Plants treated as described in Table 1 were sampled in February 1999 and assayed for GST activity.

All three sites demonstrated higher GST activities in plants surviving herbicide treatment (Table 2). At all sites, plants surviving IPU treatment possessed mean GST activities of between 2.1 and 3.4 times that of plants from untreated plots. In the two sites where diclofop was studied, plants surviving this treatment possessed mean GST activities of between 1.7 and 2.4 times those of untreated plots. Plants in plots that had been treated with fenoxaprop-P and clodinafop, but were not sprayed during the 1999 season, had average GST activities of 3.3 and 3.4 times those of untreated plots, respectively (Table 2).

Table 2. Mean GST activities in plants sampled in February 1999, expressed as µmol CDNB/min/g fresh biomass. n=10 except * n=7. Figures in brackets are ratios to untreated.

Site	Untreated	IPU	Diclofop	Clodinafop	Fenoxaprop -P
1	0.44	0.90 * (2.05)	0.74 (1.68)	--------	--------
2	0.18	0.62 (3.44)	--------	0.62 (3.44)	0.60 (3.33)
3	0.34	0.99 (2.91)	0.80 * (2.35)	--------	--------

Time course of GST activity

Plots at site 2 were resampled four times between March and May 1999. Data for mean GST activities are shown in Figure 1.

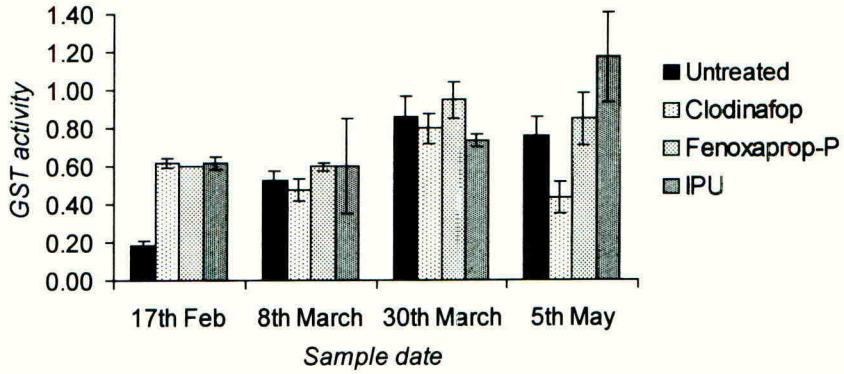

Figure 1. GST activity (µmol CDNB/min/g fresh biomass) for black-grass populations from four different field trial plots. Sampling on four dates between February and May. Values are means ± SE, where n=10.

Detection of polypeptide using anti-GST antibodies

Results are shown in Figure 2.

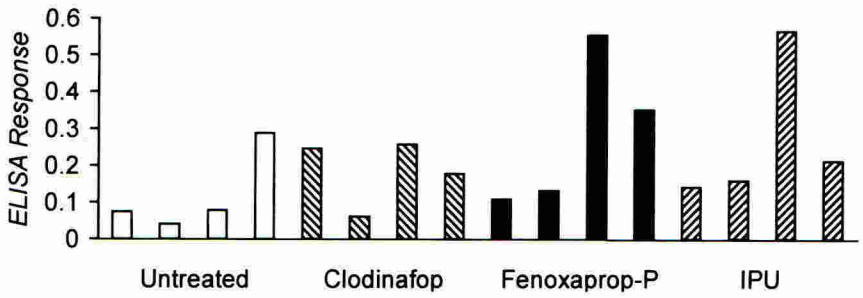

Figure 2. ELISA response for extracts from four plants from each treatment plot (site 2, February 1999). Response is for 1 μg total protein loading per well. Each bar represents a single black-grass plant. ELISA response is expressed as optical density (arbitrary units) at 405 nm.

DISCUSSION

At all sites the range of GST activities from untreated plots showed some degree of crossover with the ranges for treated plots. However, the lower GST activities in the untreated plots were absent from treated plots. This would be expected if the treated plots contained populations 'selected' from the untreated plots due to their higher GST activities. Although there is evidence that fenoxaprop-P can be metabolised by GSTs in some species (eg. Tal *et al.*, 1993) there is no evidence that clodinafop, diclofop or IPU are metabolised by this enzyme.

In sites 1 and 3 raised activities could be a result of herbicide treatment as opposed to innately higher GST activities in the population. However, site 2, which did not receive herbicide treatment during the season of study, also showed raised activities. Populations in site 2 are a result of repeated use of single types of herbicide year after year. These results suggest that populations surviving herbicide treatment consisted of plants with higher GST activities, even with respect to herbicides that are not metabolised by this enzyme family. Hence, the GST activity profile of a black-grass population may allow a prediction of how well that population will be controlled by herbicide. Further sampling of site 2 (in March and May) indicated that the initial observed difference in GST activities between treated and untreated plots (in February) was not present later in the season. GST activities in untreated plots were similar to those in treated plots by early March, and the activities in untreated plots remained similar to treated plots on subsequent samplings. This suggests that any prediction of herbicide resistance utilising GST activities would have to be carried out early in the season. These raised GST activities in untreated plots may also provide some insight into the reduced efficacy of some graminicides in the control of black-grass when applied in the spring (Milner *et al*, 1999).

Detection of polypeptides using antisera raised against a purified GST subunit from black-grass indicated that raised GST activities may be a result of the presence of more GST polypeptide. ELISA detection of immunoreactive material from four plants

from each plot at site 2 indicated that all but one plant from treated plots had more detectable polypeptide than thee of the four plants from untreated plots.

From this study it is evident that either GST activity (against CDNB) or ELISA detection using anti-GST antisera may be used to predict the response of a population to herbicide treatment. As activity studies take less than one day, and ELISA study can be completed within thee days, these tests would give results very quickly. In addition, as the tests would be carried out as soon as black-grass plants were at the two-leaf stage, results could be obtained before application of post-emergence herbicides. This would allow alternative control measures to be adopted where herbicide resistance was indicated.

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Activity of tepraloxydim (BAS 620H), a new cyclohexanedione herbicide, on herbicide-resistant black-grass (*Alopecurus myosuroides*)

R E Ruske
Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, UK

S R Moss
IACR-Rothamsted, Harpenden, Herts AL5 2JQ, UK

ABSTRACT

Experiments were conducted to determine the efficacy of tepraloxydim (BAS 620H), a new cyclohexanedione herbicide, on a range of black-grass (*Alopecurus myosuroides*) populations, with contrasting resistance characteristics. In glasshouse dose response assays and petri-dish experiments, there was evidence that target site resistance (insensitive ACCase) reduced the activity of tepraloxydim. However, the effects of target site resistance on tepraloxydim activity were much less than those recorded in previous experiments with a range of other ACCase inhibiting herbicides. There was no evidence that tepraloxydim was affected by any other resistance mechanisms present e.g. enhanced metabolism. In an outdoor container experiment, the efficacy of tepraloxydim was reduced by the presence of target site resistance, although at the proposed field rate (50g a.i./ha) control of all populations, regardless of resistance mechanism, was good (> 83%). Tepraloxydim gave more consistent control of resistant black-grass than any of the other ACCase inhibiting herbicides tested (cycloxydim, fluazifop and propaquizafop).

INTRODUCTION

Tepraloxydim (BAS 620H) is a cyclohexanedione ("dim") herbicide being developed for use in broad leaved crops by BASF (Kibler, 1999). One of the main target weeds for this herbicide is black-grass (*Alopecurus myosuroides*).

Herbicide-resistant black-grass occurs at least on 746 farms in England and in several other European countries (Moss *et al.*, 1999). Mechanisms of resistance include enhanced metabolism and target site resistance (insensitive ACCase) (Cocker *et al.*, 1999). Previous studies (Moss & Clarke, 1995) demonstrated that target site resistance gave a very high degree of resistance at the whole plant level to a range of ACCase inhibiting herbicides. The objective of this study was to evaluate tepraloxydim activity on resistant black-grass.

MATERIALS AND METHODS

The six black-grass populations used in both the glasshouse and container studies encompass all the known mechanisms of resistance present in UK black-grass (Table 1). Four additional populations (Beds. E1 1998, Oxford AA1 1994, Northants. F1 1998, and Lincs. O1 1998),

which all possess target site resistance (insensitive ACCase), were included in one petri-dish assay.

Table 1. Resistance mechanisms of populations used in glasshouse, petri-dish and outdoor container experiments. (Cocker *et al.*, 1999; Price *et al.*, 1997)

	Enhanced metabolism	Target site (ACCase)	Other mechanisms (uncharacterized)
Rothamsted 1996	S	S	No
Peldon Hams 1996	✓✓✓	No	No
Notts. A1 1993	✓	in 90% plants	No
Lincs. E1 1994/95	✓✓	in 5% plants	Yes
Claycroft 1997	✓	No	Yes
Warwicks. G1 1997	✓?	Resistant to AOPP but not CHD herbicides?	No?

S = susceptible. ✓, ✓✓, ✓✓✓ = show enhanced metabolism to a low, medium and high degree. AOPP = aryloxyphenoxypropionates, CHD = cyclohexanediones.

Glasshouse dose response assay

Six black-grass populations were treated with tepraloxydim applied post-emergence at 10 doses (range 0.78 – 400 g a.i./ha) to plants grown from seeds in 5cm pots containing compost. The experiment comprised a fully randomized design with 20 pots (1 plant/pot) per dose. There were also 40 untreated pots for each of the six populations. Treatments were applied on 13 October 1998 when the plants were at the 3 – 3 ½ leaf stage. The herbicide was applied using a laboratory sprayer delivering 243 litres water/ha at 210kPa through a single Teejet 110015VK ceramic nozzle. On 4 November 1998, 22 days after spraying, herbicide activity was recorded by assessing the foliage fresh weight for each individual pot. Dose response data was analysed using a logistic relationship between foliage fresh weight and $\log_{10}$ dose, and ED_{50} values (herbicide dose required to reduce fresh weight by 50% relative to untreated) determined.

Petri-dish experiments

Two experiments were conducted in petri-dishes to determine the response of tepraloxydim in comparison with the other cyclohexanedione herbicides, cycloxydim and sethoxydim. In the first experiment the response of two populations, Rothamsted (susceptible) and Notts. A1 (target site resistance) to tepraloxydim, cycloxydim and sethoxydim was investigated. For each population at each dose, 50 seeds were placed in each of two replicate petri-dishes containing three cellulose and one glass fibre filter paper. The dose range for each herbicide comprised 0.1, 1, 5, 10, 100 and 1000 ppm. This was achieved by completing a dilution series of the herbicide using a KNO_3 solution (2 g/litre). Seven ml of each herbicide solution was added to respective petri-dishes, on 27 November 1998. For each population there were also six control dishes (two per herbicide) in which only KNO_3 solution was added. All petri-dishes were incubated at settings of 17^0C 14 hour day; 11^0C 10 hour night, for 17 days. Dishes were then assessed by recording the shoot length for each germinated seed.

In the second experiment, seven populations were used. These included Rothamsted 1996 (susceptible), Notts. A1 1993, and Warwicks. G1 1997, which were also used in the glasshouse and container experiments. In addition four other populations were used, which have shown evidence of target site resistance to ACCase inhibitors in previous experiments (Oxford AA1 1994, Lincs. O1 1998, Northants. F1 1998 and Beds. E1 1998). The response of these seven black-grass populations to tepraloxydim (0.01, 0.1, and 1ppm) cycloxydim (5ppm) and sethoxydim (10ppm) was determined using the same techniques as described in the previous experiment. The experiment was set up on 14 January 1999, and dishes assessed on 1-3 February 1999, 18-20 days after the addition of herbicide. The main assessment consisted of recording the number of seeds with shoots > 1cm. In addition, the shoot lengths of the Rothamsted and Notts. A1 populations treated with tepraloxydim, and the control, were assessed.

Outdoor container experiment

The response of six black-grass populations to tepraloxydim, cycloxydim + "Actipron", fluazifop-P-butyl + "Partna", and propaquizafop was assessed in an outdoor container experiment to simulate field conditions. The populations were the same as those used in the glasshouse experiment. The seeds (200-350 per container) were incorporated into the top 5cm of a silty loam/grit mix in separate plastic containers (27x18x10cm deep) on 22 September 1998, and placed in an outdoor sand bed. The experiment comprised a randomized block design with three replicates, and two untreated containers per replicate for each population. Details of herbicide doses are given in Table 5. Treatments were applied post-emergence on 17 November 1998, when black-grass growth stage was 1-2 tillers, using a laboratory sprayer delivering 277 litres water/ha. Herbicide activity was assessed between 15 - 17 February 1999, by recording the number of plants that were alive in each container.

RESULTS

Glasshouse dose response assay

Tepraloxydim showed high levels of activity against black-grass in the glasshouse. Over 90% reduction in foliage weight was achieved in five populations by 25 g a.i./ha. Notts. A1, the population with the highest level of target site resistance, was significantly ($P \leq 0.05$) less sensitive than the other populations (Table 2). However, the Resistance Index for Notts. A1 (4.6) was much smaller than that shown for other ACCase inhibiting herbicides in previous experiments with the same population (Moss & Clarke, 1995), where the Resistance Index for sethoxydim was over 400, and over 100 for fenoxaprop. Warwicks. G1 was also statistically less sensitive ($P \leq 0.05$) than the standard population. However the Resistance Index of 1.7 (Table 2) is less than two fold, and is unlikely to have any appreciable impact on field efficacy.

Table 2. Response of six black-grass populations to tepraloxydim in a dose response assay.

Population	$Log_{10} ED_{50}$	ED_{50} (g a.i./ha)	Resistance Index
Rothamsted	0.625	4.2	1.0
Peldon Hams	0.786	6.1	1.5
Notts. A1	1.283	19.2	4.6
Lincs. E1	0.787	6.1	1.5
Claycroft	0.698	5.0	1.2
Warwicks. G1	0.857	7.2	1.7
S.E. ±	0.056	-	-
L.S.D. ($P \leq 0.05$)	0.165	-	-

Resistance Index is the ratio of ED_{50} values relative to the susceptible Rothamsted.

Petri-dish experiments

In the first experiment tepraloxydim was very active, even at the lowest dose used (0.1ppm), to the extent that an ED_{50} value for Rothamsted could not be determined. Notts. A1 showed very high resistance to sethoxydim and cycloxydim (Table 3), but differences between Notts. A1 and Rothamsted were much smaller when they were treated with tepraloxydim.

Table 3. Response of two black-grass populations to tepraloxydim, cycloxydim, and sethoxydim in a petri-dish assay

	ED_{50} (ppm) and resistance indices (in brackets)		
Population	tepraloxydim	cycloxydim	sethoxydim
Rothamsted	< 0.1	0.06	0.17
	(n.d.)	**(1.0)**	**(1.0)**
Notts. A1	0.24	15.71	103.11
	(> 2.4)	**(262)**	**(607)**

n.d = not determined

In the second experiment the control of all the resistant populations (except Warwicks. G1), by tepraloxydim at 0.1ppm was significantly poorer ($P \leq 0.05$) than the control of the Rothamsted susceptible standard (S.E. ± 6.39) (Table 4). However at 1ppm, differences between populations were much smaller, while a dose of 0.01ppm was too low to have much effect on any population (Table 4). Both sethoxydim and cycloxydim showed large differences between the susceptible Rothamsted and resistant populations (Table 4), despite the use of higher concentrations. A dose response analysis was conducted on shoot length data for Rothamsted and Notts. A1. The $log_{10}ED_{50}$ values were: Rothamsted –1.820; Notts. A1 –0.641 with a S.E. ± 0.154. Detransformed ED_{50} values were: Rothamsted 0.015; Notts. A1 0.229 ppm giving a resistance index of 15.3. This confirms that tepraloxydim is affected by target site resistance, but to a much lesser extent than either cycloxydim or sethoxydim.

Table 4. Response of seven black-grass populations to tepraloxydim, cycloxydim and sethoxydim.

Population	% reduction in number of seeds with shoots >1cm relative to untreated				
	tepraloxydim			cycloxydim	sethoxydim
	0.01ppm	0.1ppm	1ppm	5ppm	10ppm
Rothamsted	19	75	98	100	100
Notts. A1	0	22	91	9	11
Warwicks. G1	11	56	100	100	99
Beds. E1	16	36	90	58	47
Oxford AA1	-8	3	87	12	-14
Northants. F1	6	5	95	9	7
Lincs. O1	13	-14	74	-8	-4

Outdoor Container Experiment

Tepraloxydim at 50 g a.i./ha (the proposed recommended rate) gave fairly good control of Notts. A1 (83%), a population known to show a high degree of target site resistance to many ACCase inhibiting herbicides (Table 5). However, at 25 g a.i./ha (half the proposed field rate), control of Notts. A1 was much poorer (18%). Control of all other populations by tepraloxydim at both dose rates was very good (>93%). Cycloxydim, gave good control of all populations except Notts. A1. Fluazifop and propaquizafop generally gave similar levels of control with excellent control (> 99%) of Peldon and Claycroft, mediocre (68-74%) control of Lincs. E1 and poor control of Notts. A1 and Warwicks. G1 (7-32%). Control of Warwicks. G1 by cyclohexanedione herbicides (tepraloxydim and cycloxydim), was much better than by aryloxyphenoxypropionate herbicides (fluazifop and propaquizafop). This confirms previous observations and implies that this population has a resistance mechanism specific to aryloxyphenoxypropionate herbicides. This contrasts with Notts. A1, and many other populations, which have target site resistance to both classes of herbicide.

Table 5. Efficacy of herbicides applied under simulated field conditions (outdoor containers).

Population	% reduction in surviving plant numbers relative to untreated controls				
	tepraloxydim 50g a.i./ha	tepraloxydim 25g a.i./ha	cycloxydim 150g a.i./ha	fluazifop-P 125g a.i./ha	propaquizafop 70 g a.i./ha
Rothamsted	100	100	100	100	100
Peldon	100	97	99	100	100
Notts. A1	83	18	13	24	7
Lincs. E1	98	93	93	74	68
Claycroft	100	100	100	100	99
Warwicks. G1	100	97	100	32	26
S.E. ±			3.51		
L.S.D. (P≤0.05)			9.89		

DISCUSSION

There was evidence in these experiments that the presence of target site resistance (insensitive ACCase) reduces the efficacy of tepraloxydim. However, tepraloxydim was much less affected than all the other ACCase inhibiting herbicides studied. In simulated field conditions, tepraloxydim at 50g a.i./ha gave much better control (83%) of a population with target site resistance (Notts. A1) than cycloxydim, fluazifop and propaquizafop (< 24%). Tepraloxydim gave more consistent control of a range of resistant black-grass populations than any other ACCase inhibiting herbicide. There was no evidence to indicate that tepraloxydim was affected by other mechanisms of resistance, such as enhanced metabolism (Peldon) and as yet uncharacterised mechanisms (Warwicks. G1 and Claycroft).

Thus tepraloxydim was less affected by target site resistance (insensitive ACCase), than the other ACCase inhibiting herbicides studied. However, its relative activity in the field is likely to be strongly influenced by the dose and possibly the size of black-grass plants at time of application. Tepraloxydim should be used within a resistance management strategy, which includes cultural methods and herbicides with different modes of action, to minimise the further evolution of resistance.

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Genetic variation and relationships of herbicide-resistant and -susceptible biotypes of *Lindernia micrantha*

H Shibaike, K Itoh
National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604, Japan

A Uchino
Tohoku National Agricultural Experimental Station, Omagari, Akita 014-0102, Japan

ABSTRACT

The process of resistance evolution to ALS-inhibitors in *Lindernia micrantha* populations was investigated using molecular markers. The results indicated that populations of resistant biotypes contained less variation than those of susceptible biotypes, and that in some cases the populations of resistant biotypes were nested in the populations of susceptible biotypes. We concluded that multiple founding events of resistant biotypes occurred in *L. micrantha* populations in Japan.

INTRODUCTION

Recently (1996-97), *Lindernia micrantha* (azetogarashi) biotypes resistant to the acetolactate synthase (ALS) inhibitor herbicides, bensulfuron-methyl and pyrazosulfuron-ethyl, were reported from four prefectures in Honshu island, Japan (Itoh *et al.*, 1999). We can hypothesize two evolutionary scenarios for to the rapid spread of ALS-inhibitor resistant azetogarashi. The first hypothesis is that there has been a single founding event in which the resistant biotypes developed at a particular locality and spread rapidly into the other areas by unintentional seed movement during agricultural activities. The second hypothesis is that there have been multiple founding events in which the resistant biotypes developed at several localities and then spread locally by seed and/or pollen dispersal mechanisms. One method to distinguish these two mechanisms is to collect pairs of resistant and susceptible biotypes from each of the localities and assess the relationships among these biotypes. It is also valuable to clarify the above hypotheses in order to understand the likelihood and speed of evolution of herbicide resistance, because the initial resistance gene frequency is one of the essential parameters in the models that describe the rate of emergence of herbicide resistance (cf. Table 8.2 in Cousens & Mortimer, 1995). In this study, we used a molecular marker technique to estimate the genetic variability in 12 populations of azetogarashi including the biotypes resistant and susceptible to the ALS-inhibitors. Based on these estimates, we perform clustering analysis to obtain insight into the relationships among azetogarashi populations in Japan with special regard to the origin of resistant biotypes.

MATERIALS AND METHODS

Azetogarashi is an annual weed that occurs in paddy fields or mesic places in Japan. Twelve populations of azetogarashi (AKT, ank, ans, aom, ash, AST, knb, KTM, KTN, YCG, YSS and ytc) were selected from Akita, Kyoto, and Yamagata prefectures. Their localities are shown in Figure 1. Three to eight individuals (total 69 plants) were randomly collected from each

population in July 1998. We carefully discriminated the biotypes resistant or susceptible to ALS-inhibitors by interviewing farmers about the history of herbicide use on their fields before collecting samples. In addition, resistance or susceptibility was determined using the diagnostic method of Uchino *et al.* (1999).

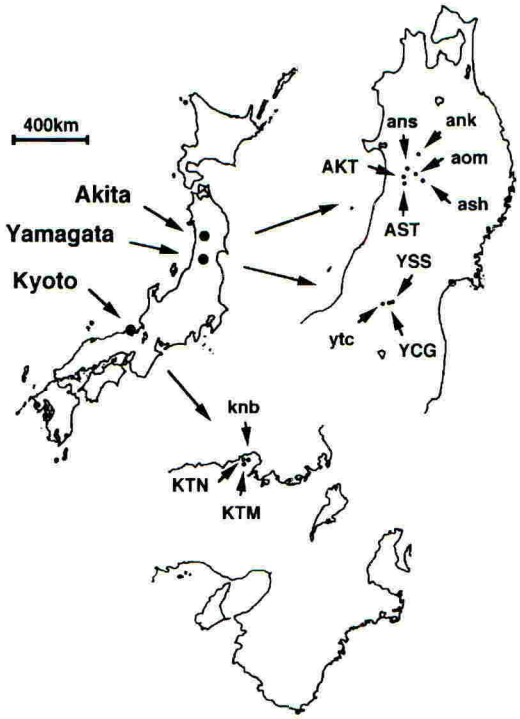

Figure 1. Localities of 12 population samples of *L. micrantha*. Resistant biotypes in capitals, susceptible biotypes in lower case.

To estimate the genetic variability of the 12 populations, we used inter-simple sequence repeat (ISSR) markers (Zietkiewick *et al.* 1994). ISSR markers were generated from single-primer polymerase chain reaction (PCR) amplifications where the primer is designed from di- or trinucleotide repeat motifs with a 5' or 3' anchoring sequence of one to three nucleotides. Total DNA was isolated from frozen leaves using a CTAB procedure (Weising *et al.*, 1991). Nine oligonucleotide primers (Table 1) were synthesized according to Tsumura *et al.*, (1996) and Wolfe *et al.* (1998). The conditions for PCR amplifications also followed the above authors' protocols except the thermal cycler (GeneAmp PCR system 9700; Perkin Elmer). Amplified products were separated on 2 % agarose gels (NuSieve 3:1; FMC) in TAE buffer, stained with ethidium bromide, and photographed under UV light using a red filter. The ISSR phenotype of each individual was transformed into a binary character matrix in which each locus was represented by an individual character (1: band present; 0:band absent). Simpson's index of diversity (1-D_S), which is the complement of Simpson's index D_S, was used to estimate the genetic diversity of each population detected by each primer (Simpson 1949). The clustering analysis of 69 individuals was made using NEIGHBOR program of PHYLIP ver. 3.57c (Felsenstein, 1993). The distance matrix among individuals used in NEIGHBOR was estimated as follows. A simple matching coefficient of similarity (S) was used to calculate

the proportion of bands shared between any two ISSR phenotypes by SIMQUAL program of NTSYS-pc (Rohlf, 1993). Pairwise distance (D_g) was calculated as 1-*S*.

RESULTS

The nine primers used yielded a total of 23 polymorphic loci. The number of polymorphic loci and the number of ISSR phenotypes detected by each primer varied from one ((AG)$_8$T and (AG)$_8$TA) to five ((CA)$_8$G) and from two ((AG)$_8$T and (AG)$_8$TA) to eight ((AC)$_8$C and (CA)$_8$G) (Table 1). The combination of these nine primers recognized 40 different ISSR phenotypes out of 69 individuals. The mean number of ISSR phenotypes across nine primers and Simpson's indices of diversity were variable among the populations (Table 1). In the populations of resistant biotypes (KTM, KTN, YCG, YSS, AKT, and ASS), the mean number of ISSR phenotypes was almost one, and the Simpson's indices of diversity were nealy zero. Whereas, in the populations of susceptible biotypes (knb, ytc, aom, ank, ans, and ash), both parameters varied from one to 2.3 and from zero to 0.21. Their highest values did not differ greatly among the populations from the three prefectures (i.e., 1.7 and 0.15 in ans; 2.3 and 0.21 in knb; 1.9 and 0.11 in ytc). A UPGMA (unweighted pair-group method using arithmetic averages) dendrogram for 69 individuals based on 23 polymorphic loci is shown in Figure 2. The dendrogram consisted of two major clusters. The first cluster contained Kyoto populations, and the second one contained Akita and Yamagata populations. The second cluster also consisted of two major clusters, however Akita and Yamagata populations were not assembled in each of these clusters. As expected from the above results, the resistant biotypes in the populations were tightly clustered with each other in most cases. Whereas, the susceptible biotypes appeared in different major clusters (e.g., ans and ytc). In the cluster comprised of Kyoto populations, two populations of resistant biotypes (KTM and KTN) were clearly nested in the population of susceptible biotypes (knb).

DISCUSSION

ISSR markers identified considerable genetic variation in azetogarashi populations in Japan. The populations of resistant biotypes showed less within-population diversity than those of susceptible biotypes in the number of ISSR phenotypes and Simpson's index of diversity (Table 1). Previous isozyme electrophoresis studies of resistant populations have also shown that resistant biotypes possess a much narrower genetic base than adjacent susceptible populations (Warwick & Black 1986). In the present study, three out of six populations of resistant biotypes were monomorphic. This is due to the "founder effect" of mutants in the populations measured soon after the evolution of resistant biotypes, and is also consistent with the recent report of its emergence. Limited variability of resistant biotypes within the populations might result from the occurrence of multiple resistance alleles or the accumulation of genotypes via outcrossing with more distant plants in the same or nearby fields. As described above, there was little difference in genetic variation maintained in the populations of susceptible biotypes among three prefectures (cf. ans, knb, and ytc in Table 1). In the UPGMA dendrogram (Figure 2), close genetic relationships among Kyoto populations, and Akita and Yamagata populations were found. These results suggest that the Kyoto populations have discrete genetic bases from Akita and Yamgata populations, and also that Akita and Yamagata populations have similar genetic bases. Therefore, the resistant biotypes are likely

Table 1. The number of ISSR phenotypes in 12 populations of *L. micrantha* detected by each of the nine primers, and Simpson's index of diversity for each population. The numbers of the individuals examined for each population was shown in parenthesis.

Primers	Number of ISSR phenotypes											
	Kyoto Pref.			Yamagata Pref.			Akita Pref.					
	KTM (5)	KTN (4)	knb (8)	YCG (5)	YSS (5)	ytc (8)	AKT (5)	AST (5)	aom (5)	ank (8)	ans (8)	ash (3)
$(AG)_8T$	1	1	2	1	1	1	1	1	1	2	1	1
$(AG)_8TA$	1	1	2	1	1	1	1	1	1	1	1	1
$(AC)_8C$	1	1	1	1	1	3	1	1	2	3	3	1
$(AC)_8T$	1	1	3	1	1	1	1	1	1	1	2	1
$(CA)_8G$	1	1	5	2	1	3	2	1	1	2	2	1
$(CA)_8TA$	1	4	1	1	1	2	1	1	1	1	2	1
$(CA)_6RY$	1	1	4	2	1	2	1	1	1	1	1	1
$(GT)_6AY$	1	1	1	1	1	3	1	1	2	2	2	1
$CAA(GA)_5$	1	1	2	1	1	1	1	1	1	1	1	1
Mean	1	1.3	2.3	1.2	1	1.9	1.1	1	1.2	1.6	1.7	1
Simpson's index of diversity	0	0.05	0.21	0.05	0	0.11	0.01	0	0.04	0.07	0.15	0

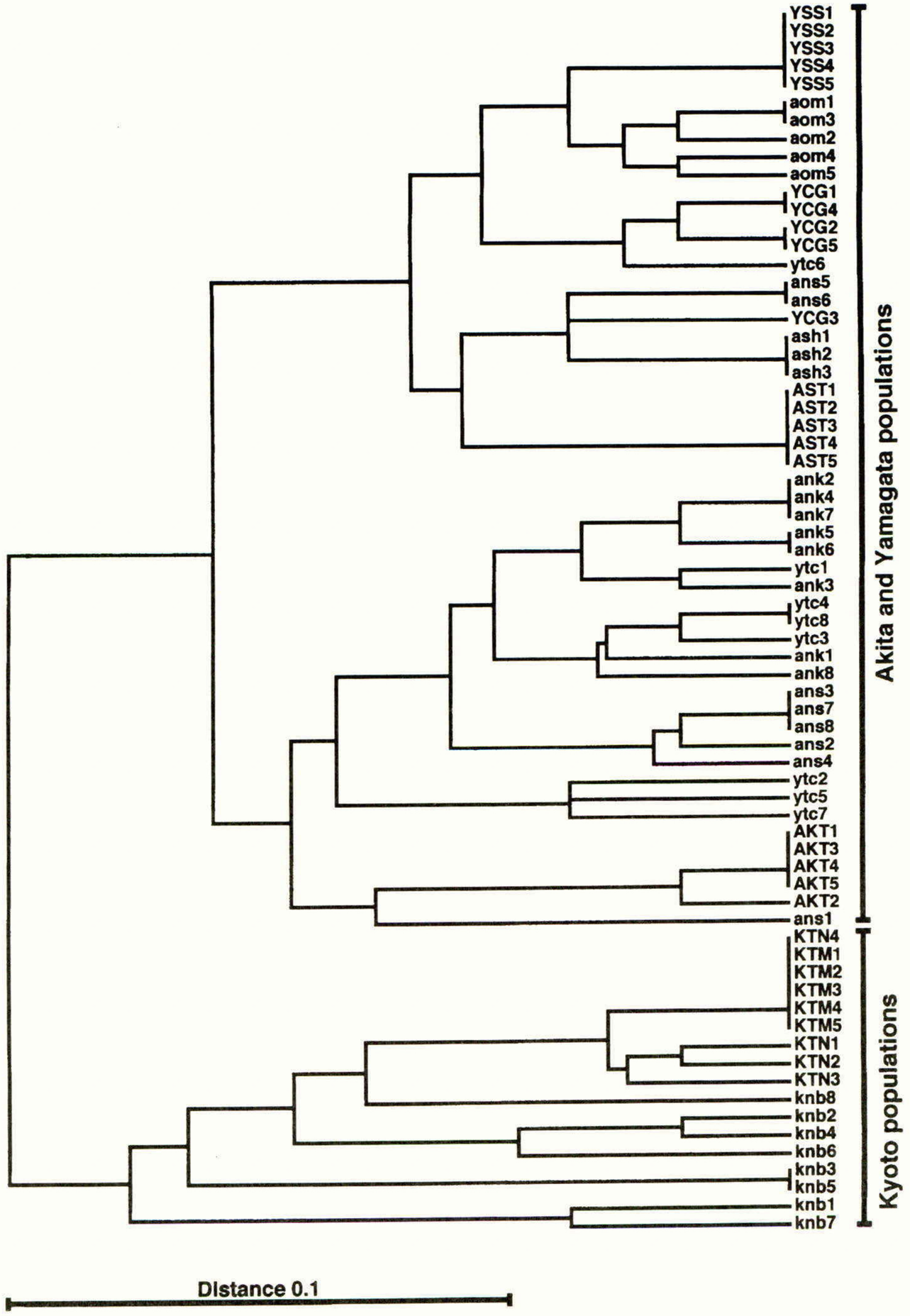

Figure 2. UPGMA dendrogram of 69 individuals of *L. micanthra*

to occur spontaneously in these two regions (i.e., Kyoto, and Akita and Yamgata prefectures). However, present results do not allow discrimination between multiple independent origin of resistance versus spread via gene flow in Akita and Yamagata populations. Acetolactate sysnthase (ALS) is the first enzyme in the biosynthetic pathway of the branched chain amino acids valine, leucine, and isoloicine (Umbarger, 1978). Five highly conserved amino acid residues within the ALS enzyme primary structure have been reported to account for naturally or in vitro selected resistance to ALS-inhibitors in plants when changed from the wild-type amino acid (cf. Fig. 1 in Wright *et al.*, 1998). Our preliminary experiments have also revealed that the resistant biotypes of azetogarashi are due to the mutation in one of the five residues (data not shown). In the next step, we will try to examine the possibility that multiple founding events with multiple resistant alleles occurred, and different resistant mutations were then spread locally by seed and pollen dispersal (cf. Guttieri *et al.*, 1995).

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