PROJECT REPORT No. 279

DETECTION OF HERBICIDE RESISTANCE IN BLACK-GRASS, ITALIAN RYE-GRASS AND WILD-OAT AT ALL GROWTH STAGES

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DETECTION OF HERBICIDE RESISTANCE IN BLACK-GRASS, ITALIAN RYE-GRASS AND WILD-OAT AT ALL GROWTH STAGES

PART 1: ASSAY DEVELOPMENT

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ABSTRACT

Herbicide resistant black-grass was first detected in 1982, since then herbicide resistance has also been confirmed in Italian rye-grass and wild-oat. A need exists for a rapid, reliable and inexpensive method for the detection of herbicide resistance during the development of the crop. The aim of this HGCA funded project was to develop a rapid bioassay for the detection of herbicide resistance in black-grass, Italian rye-grass and wild-oat at various growth stages. Confirmed susceptible (S) and resistant (R) biotypes of each species were used to define suitable dose-ranges to discriminate between R and S glasshouse grown propagules of each species. Soil was removed from the root systems of seedlings or rooted tillers, these were subsequently trimmed to 5 cm shoot and root prior to immersion in a range of herbicide doses. Dose-response curves were fitted and discrimination between biotypes was based on I_{50} values of length and fresh weight of new shoot growth.

Assays conducted on seedlings and tillers of all species were able to discriminate between R and S biotypes over a range of herbicide doses. Clear discrimination between Peldon (R) and Rothamsted (S) biotypes of black-grass were achieved with fenoxaprop-P-ethyl, isoproturon and chlorotoluron. Differences between two Italian rye-grass biotypes, Clev (R) and Halja (S) were achieved over a range of doses of fenoxaprop-P-ethyl and isoproturon. Fenoxaprop-P-ethyl was also used to discriminate between R (T/11 and T/41) and S (LLUD) biotypes of wild-oat. Fresh weight was found to be the most accurate indicator of propagule response to herbicide dosage, especially for those assays conducted with Photsystem II inhibitor herbicides. Resistance to fenoxaprop-P-ethyl was detected in all species, even where resistance was partial due to enhanced metabolism.

The assays are suitable for detecting herbicide resistance until nodes are detectable on the weed species. Results obtained for resistance detection assays at later growth stages utilising cut stem node segments proved unsuccessful and it was concluded that tests using collected seeds would give more reliable results.

The robustness of the assays under contrasting temperature, humidity and light were examined using seedlings of S and R biotypes. Distinction between R and S biotypes was achieved under all conditions the assays were subjected to. The study also examined the effect of storage on propagules. Seedlings and tillers of all biotypes were packaged and stored at $1^{o}C$ for 31 and 29 days respectively and were found to still be viable after these time periods.

SUMMARY

Introduction

Herbicide resistance is the inherited ability of a weed to survive treatment by herbicide and often results from over-reliance on herbicides with a single mode of action or common degradation pathway. Black-grass resistance to chlorotoluron and isoproturon was first confirmed in the UK in 1982. Herbicide resistance in black-grass results commonly, but not exclusively, from enhanced metabolism due to elevated levels of mixed function oxidases in resistant plants. This mechanism explains cross-resistance to herbicides with other modes of action and different chemistries. Populations have also been confirmed with target site resistance (insensitive acetyl co-enzyme A carboxylase or ACCase) to fenoxaprop-P-ethyl and other ACCase inhibitors. Black-grass populations have also been confirmed with additional, but as yet undefined, mechanisms of resistance to several ACCase inhibitor herbicides including fenoxaprop-P-ethyl and clodinafop. In addition to herbicide resistant black-grass, populations of wild-oat and Italian rye-grass have been confirmed with varying levels of herbicide resistance adding to the problems of weed management on cereal farms.

Herbicide treatments may fail for a variety of reasons and confirmation that herbicide resistance is involved requires a reliable test. The most common approach is to grow plants from seed collected from the suspected resistant (R) population and compare its response to herbicide treatment with that of plants from confirmed susceptible (S) and populations. Cheaper and more rapid testing of seeds can be made in a Petri dish assay. A need exists for a rapid, reliable and inexpensive method for the detection of herbicide resistance during crop development. The aim of this study was to develop a rapid bioassay for the detection of herbicide resistance using vegetative propagules of black-grass, Italian rye-grass and wild-oat at various growth stages.

Dose response assays

Seeds of confirmed resistant R and S, to various herbicides, of black-grass, Italian rye-grass and wild-oat were used in the assays. The biotypes used and resistance mechanisms are shown in Table 1.

Table 1. Resistance mechanisms and origin of seeds for species and biotypes used in assays for the detection of herbicide resistance.

Species	Biotype	R/S	Resistance mechanism	Collected
Black-grass	Peldon	R	Enhanced metabolism	Essex 1996
	Rothamsted	S	-	Rothamsted 1999
Italian rye-grass	Clev	R	Enhanced metabolism	Wiltshire 1997
	Halja	S	-	Commercial 1997
Wild-oat	T/41	R	Target site (insensitive ACCase)	Essex 1995
	T/11	R	Enhanced metabolism	Essex 1995
	LLUD	S	-	Long Ashton 1995

All seeds were germinated in peat based compost and pricked out into 9 cm diameter pots filled with sandy loam soil amended with grit. All plant material was glasshouse grown until harvested for use in bioassays.

Assay propagules and conditions

Seedlings tests were conducted on all species of seedlings at three different growth stages (2-3 leaf, 4 leaf – 1 tiller, and 2-3 tiller) whilst tiller tests were conducted with black-grass and Italian rye-grass on plants with 5 – 10 tillers and on wild-oat plants with 3 – 5 tillers. For seedling tests, all soil was carefully removed from the root system prior to trimming the seedlings to 5 cm shoot and root length. Trimmed seedlings of R and S biotypes were then placed in glass vials containing a range of herbicides and doses (Table 2). Tillers were carefully teased from the mainstem and treated as seedlings. Assays were arranged as randomised blocks, and conducted in controlled environment (CE) at 17 ^{o}C 16 hour day, 10 ^{o}C 8 hour day. Propagules were re-trimmed after 24 hours. Length and fresh weight of new shoot growth were assessed seven days after treatment for all tests apart from black-grass and Italian rye-grass tiller tests with isoproturon. Results subject to ANOVA and non-linear regression analysis with test for lack-of-fit to determine the appropriate dose response curves.

Table 2. Species, growth stages and herbicides on which dose response assays were undertaken.

Species	Test	Herbicides
Black-grass	ST[1] TT[2]	fenoxaprop-P-ethyl, isoproturon, and chlorotoluron
Italian rye-grass	ST TT	fenoxaprop-P-ethyl, isoproturon,
Wild-oat	ST TT[3]	fenoxaprop-P-ethyl

[1] ST – seedling test. [2] TT – tiller test. [3]T/41 target site resistant wild-oat biotype.

Results

Example: Italian rye-grass seedling test at 2-3 leaf stage of growth treated with fenoxaprop-P-ethyl and isoproturon.

Table 3. Doses of herbicide required to reduce assessed growth parameters by 50% (I_{50}) and resistance indices (RI) in Italian rye-grass.

Herbicide	Assessed parameter	I_{50}[1]	RI[2]
Fenoxaprop-P	fresh weight	R – 0.53	44
		S – 0.012	
	length	R – 0.32	29
		S – 0.011	
Isoproturon	fresh weight	R – 0.36	3.6
		S – 0.10	
	length	R – 0.33	3.7
		S - 0.09	

[1] De-transformed, mg a.i. L^{-1}.
[2] I_{50} R/ I_{50} S

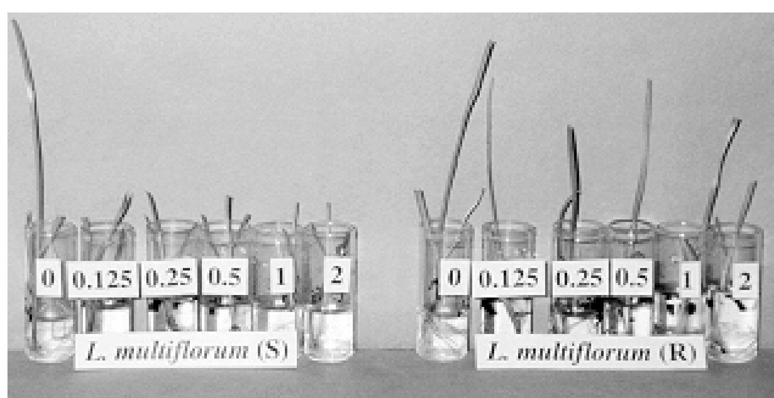


Photo 1. Seedling test with Italian rye-grass at the 2-3 leaf stage treated with a range of doses (0, 0.125 0.25, 0.5, 1 and 2 mg a.i. L^{-1}) of fenoxaprop-P-ethyl (Halja - R left, Clev - S right) seven days after treatment.

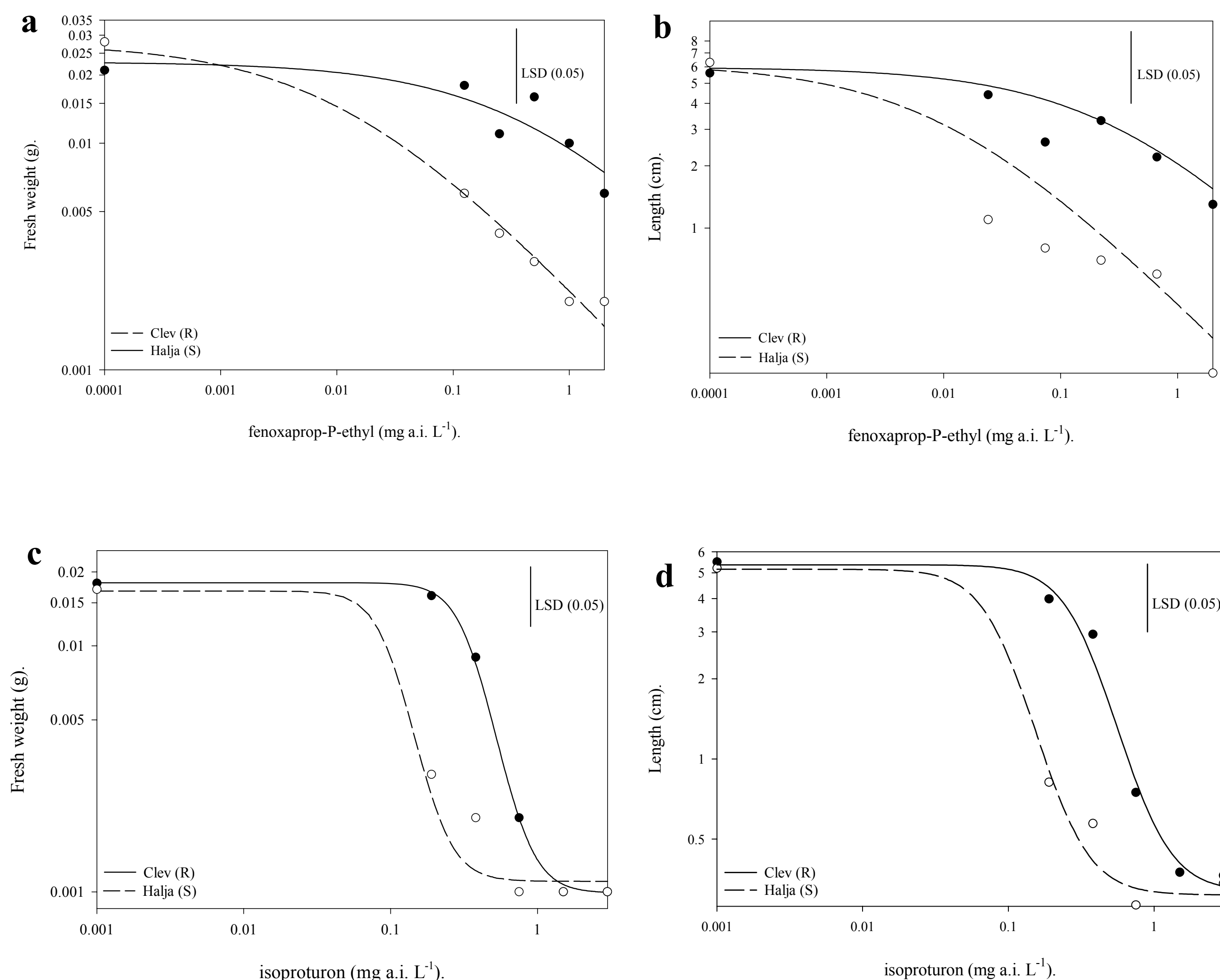


Figure 1. Fresh weights (a and c) and lengths (b and d) of new shoot growth of trimmed seedlings at the 2-3 leaf stage of Italian rye-grass treated with fenoxaprop-P-ethyl (a and b) and isoproturon (c and d).

De-transformed I_{50} values and resistance indices for dose-response assays conducted on Italian rye-grass seedlings at the 2-3 leaf stage treated with fenoxaprop-P-ethyl are presented in Table 3 and the fitted dose-response curves are shown in Figure 1 a-d. Clear discrimination between R and S biotypes can be seen for both assessed parameters for both herbicides. For the assays with fenoxaprop-P-ethyl, the I_{50} values are 44 and 29 times higher for the R biotype compared to the S biotype for fresh weight and length respectively. Clear differences between R and S biotypes response to a range of fenoxaprop-P-ethyl can be seen in Photo 1. For the assay conducted with isoproturon, I_{50} values for the R biotype are 3.6 and 3.7 times higher than the corresponding values for the S biotype in fresh weight and length of new shoot growth respectively.

Table 4. Summary of dose-response assays where there were significantly different responses to a range of herbicide doses between R and S biotypes in length or fresh weight of new shoot growth.

Species	Herbicide	Growth stage	Growth parameter
black-grass	fenoxaprop-P	2-3 leaf	F wt and length
		2-3 tiller	F wt and length
		Tiller test	F wt and length
	isoproturon	2-3 leaf	F wt only
		2-3 tiller	F wt and length
		Tiller test	F wt and length
	chlorotoluron	4 leaf – 1 tiller	F wt and length
		Tiller test	F wt and length
Italian rye-grass	fenoxaprop-P	2-3 leaf	F wt and length
		4 leaf – 1 tiller	F wt and length
		2-3 tiller	F wt and length
		Tiller test	F wt and length
	isoproturon	2-3 leaf	F wt only
		4 leaf – 1 tiller	F wt only
		2-3 tiller	F wt only
		Tiller test	F wt and length
wild-oat	fenoxaprop-P	2-3 leaf [1]	F wt and length
		4 leaf – 1 tiller [1]	F wt and length
		2-3 tiller [1]	F wt and length
		2-3 tiller [2]	F wt and length
		Tiller test [2]	F wt and length

[1] T/11 enhanced metabolism resistant wild-oat biotype
[2] T/41 target site resistant wild-oat biotype

Table 4 summarises the assays where there were significantly different responses to a range of herbicide doses between R and S biotypes in length of fresh weight of new shoot growth. For most of the assays there were significant differences between R and S biotypes response to herbicide treatment in both length and fresh weight of new shoot growth. The resistance assays with black-grass and Italian rye-grass employing PS II inhibitors utilise the difference in tissue damage between R and S biotypes to discriminate resistance or susceptibility, measuring the length of new shoot growth does not take into account the herbicide damage on the tissue being assessed. This is why, in a few cases, the length of new shoot growth

parameter did not discriminate between R and S biotypes when exposed to dose ranges of isoproturon, whereas the corresponding fresh weight did.

These dose-response assays have shown the ability to discriminate between S and R biotypes over a range of herbicide doses, even where the resistance is partial (enhanced metabolism resistance to ACCase inhibitors) and provide the basis for a reliable rapid and practical test for the detection of herbicide resistance (a draft test protocol is attached as Appendix B). These experiments were all conducted on glasshouse grown plants, the next stage of the project is to be undertaken at IACR - Rothamsted where the techniques will be verified on plants grown under field conditions. It is envisaged that information from the dose-response experiments will lead to a final test using a single discriminating dose to detect herbicide resistance in all three species (See validation exercise Section 6)

Stem node test

Once the first node is detectable on the tillers, they are unsuitable for use in tiller test. Hence, a new technique is required for the detection of herbicide resistance once stem elongation has begun. Stem node fragments of *Echinochloa colona* readily produced adventitious roots after 48 hours with the node immersed in water, stem node segments of R and S *E. colona* were then immersed in herbicide solution and fresh weight and length of new shoot growth after 10 days were used to discriminate between R and S biotypes. This methodology was employed in black-grass, Italian rye-grass and wild-oat but was unsuccessful. Wild-oat stem node segments were found not to form viable propagules, even after three weeks. Black-grass and Italian rye-grass did form roots and shoots but the process was low yielding and their response on exposure to herbicides were unreliable.

Due to the low yield of stem node propagules suitable for bioevaluation and their extreme variability when assayed, resistance would be more reliably detected using assays conducted on seeds of suspected R populations, such as the Rothamsted Rapid Resistance Test. By the time 2 or 3 nodes are detectable on a weed species it is too late to take remedial action, thus there is no penalty in waiting for the results of seed assays.

Robustness of assays under contrasting temperature, humidity and light

Single dose assays were conducted with all species using representative herbicide modes of action over contrasting temperature, humidity and light conditions. These were conducted with young seedlings (2-3 leaf) only, on the premise that they would be the propagules which would be most affected by change in environmental conditions. These assays were conducted on all species and used a single discriminating dose of fenoxaprop-P-ethyl (all species) and

isoproturon (black-grass and Italian rye-grass). Assays were duplicated and carried out in CE where one environmental parameter could be varied between two CE cabinets whilst the other conditions remained constant, assay conditions can be seen in Table 5.

Table 5. Controlled environment conditions employed to test the robustness of the assays.

Variable parameter	Contrasting levels	Constants [1].
Light	200 µmol m^{-2} s^{-1} vs	Temperature (17 °C d, 10 °C n)
	100 µmol m^{-2} s^{-1}	Humidity (75 % RH)
Temperature	17 °C day, 10 °C night vs	Light (200 µmol m^{-2} s^{-1})
	10 °C day, 5 °C night	Humidity (75 % RH) [2]
Humidity	90 % RH vs 50 % RH	Temperature (17 °C d, 10 °C n)
		Light (200 µmol m^{-2} s^{-1})

[1] 16 hour day, 8 hour night.
[2] humidity adjusted at low temperature regime to take into account vapour pressure deficit.

The results showed that for all the assays conducted significant differences were detected between R and S biotypes under every individual assay condition. Although in two cases with Italian rye-grass assays there were significant light effects, however at each light level discrimination between R and S biotypes was achieved. It is recommended that assays are carried out at 17 °C, 16 hour day / 10 °C, 8 hour night because these were the conditions at which the herbicide doses were optimised.

Sample packaging and storage

Under certain circumstances, there may be a need to store propagules for a certain period of time before a resistance assay can be carried out. Seedlings and tillers of all species were packaged and stored for 29 and 31 days respectively and their viability after storage was assessed via assay with a discriminating dose of fenoxaprop-P-ethyl. Tillering plants of all species were trimmed to 10 cm shoot and 7 cm root, whilst seedlings at the 2-3 leaf stage were left untrimmed. The root systems of the all plants were wrapped in moist paper towel and then placed in clear plastic bags. Bags were then maintained at 1°C with 11 µmol m^{-2} s^{-1} light for 16 hours a day.

The tests demonstrated propagule viability of tillers and seedlings of all species after storage at 1°C for 29 and 31 days respectively. Seedlings at the 2-3 leaf stage were used a 'worst case' being the weakest propagules, initial observations suggest tillers may remain viable for 60 days. This method of storage was chosen because there was very little propagule growth at at 1°C and little water loss, hence, there is no need to replace the water periodically.

TECHNICAL DETAILS

1. INTRODUCTION

Herbicide resistance is the inherited ability of a weed to survive treatment by herbicide (Gressel, 1991) and often results from over-reliance on herbicides with a single mode of action or common degradation pathway. So far, herbicide resistance has been detected in 49 countries world-wide (Heap, 2000). Herbicide resistance has the ability to reduce yield quantity and quality through the competitive effects exerted by a weed population that would otherwise have been controlled.

Table 1. Countries where herbicide resistant black-grass, Italian rye-grass and wild-oat have been reported.

Species.	Countries where resistance reported [1].
black-grass	Israel, Germany, The Netherlands, Spain, France, Belguim, Switzerland and United Kingdom.
Italian rye-grass	USA, United Kingdom, France, South Africa and Italy.
wild-oat	Australia, South Africa, Canada, USA, United Kingdom, Chile, Belgium, France and Italy.

[1] Adapted from Heap (2000).

Herbicide resistant black-grass (*Alopecurus myosuroides*), Italian rye-grass (*Lolium multiflorum*) and wild-oat (*Avena* spp.) have been identified in many countries (Table 1).
Black-grass resistance to chlorotoluron and isoproturon was first confirmed in the UK in 1982 (Moss and Cussans, 1991). Herbicide resistance in black-grass results commonly, but not exclusively, from enhanced metabolism due to elevated levels of mixed function oxidases in resistance plants (Caseley *et al*., 1990). This mechanism explains cross-resistance to herbicides with other modes of action and different chemistries (Moss, 1990). Black-grass populations have also been confirmed with target site resistance (insensitive acetyl co-enzyme A carboxylase or ACCase) to fenoxaprop-P-ethyl (Cocker *et al*., 1999). Black-grass poulations have also been confirmed with additional, but as yet undefined, mechanisms of resistance to several ACCase inhibitor herbicides including fenoxaprop-P-ethyl and clodinafop (Hall *et al*., 1997).

In addition to herbicide resistant black-grass, populations of wild-oat and Italian ryegrass have been confirmed (Table 2) with varying levels of herbicide resistance adding to the problems of weed management on cereal farms (Clarke, 1998).

Table 2. Minimum extent of herbicide resistance in England [1].

Species.	No. Farms	No. counties	Detected
Black-grass	746	30	1982
Italian rye-grass	30	12	1990
Wild-oat	65	19	1993

[1] Adapted from Moss *et al*,. (1999).

Table 2 shows the current status of herbicide resistant black-grass, Italian rye-grass and wild-oat in England, however, this is a minimum picture as over 90 % of farms have not had any sample tested for resistance (Moss *et al*., 1999). The International Survey of Herbicide Resistant suggests that the incidence in herbicide-resistance black-grass, Italian rye-grass and wild-oat are increasing in both number of sites and area infected (Heap, 2000).

Herbicide treatments may fail for a variety of reasons and confirmation that herbicide resistance is involved requires a reliable test. Techniques for assessing suspected herbicide resistant plants were reviewed by Moss (1995). Methods including chlorophyll fluorescence, leaf disc floatation and determination of glutathione content have had limited uptake as they tend to identify resistance to a single mode of action and may require sophisticated equipment. The most common approach is to grow plants from seed collected from the suspected resistant (R) population and compare its response to herbicide treatment with that of plants from confirmed susceptible (S) populations. This type of assay is usually conducted in a glasshouse using pot-grown plants and takes several weeks and expensive spraying equipment to complete. More rapid testing of seeds can be made in a Petri dish assay; thus Riches *et al*., (1997) confirmed the response of propanil R and S seedlings of *Echinochloa colona* (an important grass weed of rice) to pendamethalin and propanil in ten days. A limitation of all seed tests is that suspect resistant plants may take a long time to produce seed; furthermore, during collection, ripe seed from R plants will be shed and cause further problems.

There is a need for a rapid, reliable and inexpensive method for the detection of herbicide resistance during development of the crop. A method for the detection of herbicide resistant *E. colona* using vegetative propagules has been reported by Kim *et al*., (2000). The aim of this project is to evaluate these methodologies for the detection of herbicide resistance in black-grass, Italian rye-grass and wild-oat at various growth stages.

2. DOSE-RESPONSE ASSAYS

Materials and Method

Plant material

Seeds of confirmed resistant (R) and susceptible (S) biotypes, to various herbicides, of Italian rye-grass, black-grass and wild-oat were used in the assays. All seed was supplied by IACR-Rothamsted.

Table 3. Resistance mechanisms and origin of seeds for species and biotypes used in assays for the detection of herbicide resistance assays.

Species	Biotype	R/S	Resistance mechanism	Collected
Black-grass	Peldon	R	Enhanced metabolism	Essex 1996
	Rothamsted	S	-	Rothamsted 1999
Italian rye-grass	Clev	R	Enhanced metabolism	Wiltshire 1997
	Halja	S	-	Commercial 1997
Wild-oat	T/41	R	Target site (insensitive ACCase)	Essex 1995
	T/11	R	Enhanced metabolism	Essex 1995
	LLUD	S	-	Long Ashton 1995

All seeds were germinated in peat based compost and pricked out into 9 cm diameter pots filled with sandy loam soil amended with grit. All plant material was glasshouse grown until harvested for use in bioassays.

Seedling Test

2–3 leaf growth stage

R and S biotypes were sampled at the 2-3 leaf stage of growth. Plants and soil were removed from pots and most of the soil carefully washed from the root system of the seedlings. Seedlings were subsequently trimmed to leave 5 cm shoot and root and the remaining soil was carefully removed from the root system. Trimmed seedlings were then placed in 10 ml soda glass specimen tubes containing 8 ml of herbicide solution over a pre-determined range of doses (Table 4). Each bioassay was arranged as a randomised complete block design on metal trays. After treatment, trays were transferred to a controlled environment (CE) facility operating at 17 ^{o}C 16 hour day, 10 ^{o}C 8 hour night. 24 hours after treatment, seedlings were re-trimmed to 5 cm, this is to ensure that the assessed growth had occurred under the influence of herbicide.

4 leaf–1 tiller and 2–3 tiller growth stages

Experimental procedure as for 2–3 leaf growth stage, but all tillers are carefully removed from the main stem, this provides greater propagule uniformity. They are then trimmed to 5 cm shoot and root. Wild-oat assays with seedlings at these growth stages were conducted in 20 ml glass vials containing 15 ml herbicide solution.

Tiller Test

Italian rye-grass and black-grass plants were sampled between the 6–10 tiller growth stage whilst wild-oat plants were at the 3–5 tiller growth stage. Soil was carefully washed from the root system of the plants. Rooted tillers were then teased from the main stem and those at the 3–4 leaf stage were retained for use in assays. Tillers were then trimmed to 5 cm root and shoot and the remaining soil was washed from the roots. Tillers were then treated as outlined in the method for the seedling test but were conducted in 20 ml glass vials containing 15 ml herbicide solution.

Herbicides and dose ranges

The herbicides used were: fenoxaprop-P-ethyl formulated as 'Cheetah Super' (55g a.i. L^{-1}), isoproturon as 'Stress' (500g a.i. L^{-1}) and chlorotoluron as 'Dicurane' (500g a.i. L^{-1}). Table 4 shows the dose ranges at which the herbicides were employed. These dose ranges were derived from preliminary studies which aimed to maximise the differences between S and R biotypes.

Table 4. Herbicide dose ranges employed in assays and number of replications per dose

Species	Test	Herbicide	Dose range (mg a.i. L^{-1})	Replications
BG [1]	ST[2]	isoproturon	0, 0.375, 0.75, 1.5, 3 and 6.	8
IRG	ST	isoproturon	0, 0.188, 0.375, 0.75, 1.5 and 3.	8
BG	TT[2]	isoproturon	0, 0.75, 1.5, 3, 6, and 12.	10
IRG[1]	TT	isoproturon	0, 0.188, 0.375, 0.75, 1.5, 3 and 6.	10
BG	ST	chlorotoluron	0, 0.16, 0.8, 4, 20 and 100.	6
BG	TT	chlorotoluron	0, 0.16, 0.8, 4, 20 and 100.	6
BG, WO[1]	ST	fenoxaprop-P-ethyl	0, 0.024, 0.074, 0.222, 0.666 and 2.	8
IRG	ST	fenoxaprop-P-ethyl	0, 0.125, 0.25, 0.5, 1 and 2.	7
BG, IRG	TT	fenoxaprop-P-ethyl	0, 0.024, 0.074, 0.222, 0.666 and 2.	8
WO	ST[3]	fenoxaprop-P-ethyl	0, 0.25, 1.01,4.06,16.25 and 65.	6
WO	TT[3]	fenoxaprop-P-ethyl	0, 0.25, 1.01,4.06,16.25 and 65.	6

[1] WO –wild-oat, BG – black-grass and IRG – Italian rye-grass. [2] ST – seedling test and TT – tiller test. [3]Dose range employed for T/41 target site resistant wild-oat biotype

Sample assessment

All assays were assessed 7 days after treatment apart from the tiller tests with isoproturon which were sampled after 9 days (Italian rye-grass) and 11 days (black-grass). Length and fresh weight of new shoot growth from the point of trimming were measured.

Statistical Analysis

All measurements were primarily subjected to analysis of variance, having undergone a $\log_e$ transformation, in order to ascertain significant differences between S and R biotypes, for either fresh weight or shoot length of new shoot growth. The data were subjected to non-linear regression analysis with test for lack-of-fit to determine the appropriate dose-response curves which best describe the data (after Seefeldt *et al*., 1995). The dose-response curve used was,

$$y = A + \frac{C}{1+[x/I_{50}]^{B}}$$

where y is an estimate of the production due to the herbicide (measured as new shoot length or fresh weight), x is the herbicide dose, A is the high dose immunity (generally zero), C is the production of the control, B is the response rate and I_{50} is the herbicide dose which gives a 50% growth inhibition.

A resistance index (RI), defined as the ratio of the dose required to give a 50% reduction in the measured parameter of the resistant biotype relative to that of the susceptible biotype was calculated from the de-transformed I_{50} values to compare the degree of resistance displayed. All statistical analyses were conducted using Genstat 5 (Genstat 5 Committee, 1994).

Dose-response assay results

Black-grass - fenoxaprop-P-ethyl

The $\log_e I_{50}$, de-transformed I_{50} values and resistance indices (ratio of I_{50} values relative to the susceptible population) are presented in table 5.

Table 5. Doses of fenoxaprop-P-ethyl required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in black-grass.

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 leaf	R – 0.099 S – 0.021	4.7	R – 0.057 S – 0.015	3.9
2 – 3 tiller	R – 0.35 S – 0.015	23.2	R – 0.29 S – 0.01	31.8
Tiller test	R – 0.482 S – 0.008	57.4	R – 0.758 S – 0.027	28.1

[1] De-transformed, mg a.i. L^{-1}

The results of the assay conducted on the 2-3 leaf growth stage of black-grass highlight the differences in herbicide susceptibility between the two biotypes, Peldon (R) and Rothamsted (S), both in fresh weight and length of new shoot growth. There was a good correlation between fresh weight and length of new shoot growth with resistance indices being 4.7 and 3.9 mg a.i. L^{-1} respectively.

The differences between S and R biotypes are highlighted to a greater extent in both the seedling test at the 2-3 tiller growth stage and the tiller test, with their resistance indices being several orders of magnitude higher than those of the seedling test at the 2-3 leaf stage (Table 5).

Figure 1 a-f shows the fitted dose-response curves for both assessed parameters at three growth stages. The results achieved for the fresh weight parameter are mirrored by the length parameter at all growth stages. At the 2-3 leaf stage (Figure 1 a and b), the biggest differences between biotypes are apparent at the lower doses (0.024 to 0.222 mg a.i. L^{-1}) whilst for the tiller test (fig. 3 e and f), differences between biotypes are apparent over the whole dose-

range. As the growth stage of the test increases, so do the I_{50} values of the R biotypes (Table 5).

Black-grass – isoproturon

De-transformed I_{50} values and resistance indices (ratio of I_{50} values relative to the susceptible population) are presented in Table 6 and show significant differences in herbicide susceptibility between the known R biotype (Peldon) and the known S biotype (Rothamsted) in all cases apart from the seedling test at the 2-3 leaf growth stage where the difference is only detected in the fresh weight parameter.

Table 6. Doses of isoproturon required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in black-grass.

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 leaf	R – 0.382 S – 0.119	3.2	R – 0.242 S – 0.242	-
2 – 3 tiller	R – 1.33 S – 0.22	6.0	R – 0.85 S – 0.22	3.9
Tiller test	R – 8.31 S – 0.25	33.2	R – 7.24 S – 0.47	15.4

[1] De-transformed, mg a.i. L^{-1}

The resistance indices and the fitted dose-response curves (Figure 2 a-f) show that greater differences exist between the S and R biotypes in fresh weight of new shoot growth, with resistance indicies being roughly twice as high as those for length in the seedling test at the 2-3 tiller growth stage and the tiller test.

The resistance indices increase as the age of the propagule on which the assay was conducted increase. The tiller test has a resistance index of 33.2 for the fresh weight of new shoot growth, a ten-fold increase to that of the test conducted at the 2-3 leaf stage (RI 3.2).

Black-grass – chlorotoluron

Table 7 shows very large differences in the responses to doses of chlorotoluron between confirmed R biotype (Peldon) and the confirmed S biotype (Rothamsted).

Table 7. Doses of chlorotoluron required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in black-grass.

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
4 leaf – 1 tiller	R – 49.6 S – 0.11	450.9	R – 58.5 S – 0.12	487.5
Tiller test	R – 11.8 S – 0.12	98.3	R – 20.0 S – 0.65	30.8

[1] De-transformed, mg a.i. L^{-1}

The high resistance indices and fitted dose-response curves (Figure 3 a-d) indicate large differences in susceptibility to chlorotoluron between R and S biotypes. The largest differences between R and S biotypes at both growth stages and both assessed parameters occur at 0.8 and 4 mg a.i. L^{-1}.

The de-transformed I_{50} values obtained for the susceptible biotype are similar for both growth stages and for length and fresh weight of new shoot growth, however, the I_{50} values for the tiller test are lower that those in the seedling test at the 4 leaf – 1 tiller growth stage. These differences result in the resistance indices being far higher for the seedling test at the 4 leaf – 1 tiller growth stage than the tiller test in both length (487.5 and 30.8 respectively) and fresh weight (450.9 and 98.3 respectively).

Italian rye-grass – fenoxaprop-P-ethyl

Table 8 presents the de-transformed I_{50} values and resistance indices of the seedling test at 3 different growth stages and the tiller test with R and S biotypes of Italian rye-grass treated with fenoxaprop-P-ethyl. There were significant differences between the S and R biotypes in all growth stages for both length and fresh weight of new shoot growth.

Table 8. Doses of fenoxaprop-P-ethyl required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in Italian rye-grass.

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 leaf	R – 0.53 S – 0.012	44.2	R – 0.320 S – 0.011	29.0
4 leaf – 1 tiller	R – 1.386 S – 0.06	23.1	R – 0.37 S – 0.02	18.5
2 – 3 tiller	R – 0.75 S – 0.06	12.5	R – 1.32 S – 0.04	33.0
Tiller test	R – 2.83 S – 0.049	57.8	R – 5.53 S – 0.04	128.6

[1] De-transformed, mg a.i. L^{-1}

Figures 4 a-h show the fitted dose-response curves that also highlight large differences between R and S biotypes. There are differences between R and S biotypes in all tests and all doses with the exception of the lowest dose (0.024 mg a.i. L^{-1}) in the seedling tests at the 4 leaf – 1tiller and 2–3 tiller stage and tiller test. In the seedling test at the 2-3 leaf growth stage, the resistance index is higher for fresh weight than for length of new shoot growth, 44.2 and 29 respectively. The difference between R and S biotypes is also greater for the fresh weight parameter in the seedling test at the 4 leaf – 1 tiller growth stage (Table 8). However, in the case of the seedling test at the 2-3 tiller growth stage and the tiller test, the resistance indices are higher for length of new shoot growth parameter than for fresh weight.

Italian rye-grass – isoproturon

Table 9 presents de-transformed I_{50} values and resistance indices for Italian rye-grass at various growth stages treated with a range of doses of isoproturon. Fitted dose-response curves are shown as Figure 5 a-h. There were significant differences between R and S biotypes response to isoproturon at all growth stages for fresh weight of new shoot growth. However, when length of new shoot growth is the assessed parameter, significant differences only exist for the seedling test at the 2-3 leaf stage and the tiller test.

Table 9. Doses of isoproturon required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in Italian rye-grass.

Growth stage	Assessed growth parameter: Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 leaf	R – 0.36 S – 0.10	3.6	R – 0.33 S – 0.09	3.7
4 leaf – 1 tiller	R – 0.30 S – 0.066	4.5	R – 0.14 S – 0.14	-
2 – 3 tiller	R – 0.41 S – 0.23	1.8	R – 0.26 S – 0.26	-
Tiller test	R – 3.18 S – 0.41	7.8	R – 5.35 S – 0.30	17.8

[1] De-transformed, mg a.i./L^{-1}

The resistance indices for the seedling test at the 2-3 leaf growth stage were almost the same for both length and fresh weight of new shoot growth, 3.6 and 3.7 mg a.i. L^{-1} respectively. The differences between R and S biotypes were greater for the seedling test at the 2-3 leaf growth stage than for those at the 4 leaf – 1 tiller and 2-3 tiller growth stage. There were larger differences between R and S biotypes for the length (RI 17.8) and fresh weight (RI 7.8) of new shoot growth for the tiller test but these were achieved over a different dose-range than the seedling test (Table 4).

Wild-oat – fenoxaprop-P-ethyl (T/11)

De-transformed I_{50} values and resistance indices for the wild-oat biotypes LLUD and T/11 (enhanced metabolism based resistance) are presented in Table 10. In all seedling tests there were significant differences between R (T/11) and S (LLUD) biotypes for both length and fresh weight of new shoot growth.

Table 10. Doses of fenoxaprop-P-ethyl required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in wild-oat (T/11 v LLUD).

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 leaf	R – 0.079 S – 0.018	4.4	R – 0.092 S – 0.022	4.2
4 leaf – 1 tiller	R – 0.29 S – 0.044	6.6	R – 0.21 S – 0.038	5.5
2 – 3 tiller	R – 0.80 S – 0.065	12.3	R – 0.79 S – 0.15	5.3

[1] De-transformed, mg a.i. L^{-1}

These differential responses between T/11 (R) and LLUD (S) to treatment with a range of dose of fenoxaprop-P-ethyl are shown as Figure 6 a-f. There was good correlation between resistance indices for fresh weight and length of new shoot growth for the seedling tests at the 2-3 and 4 leaf – 1 tiller growth stages. I_{50} values for the R biotypes increased with the age of plant used in assay, with that at the 2-3 tiller stage (0.8 mg a.i. L^{-1}) being ten times higher than that at the 2-3 leaf stage (0.079 mg a.i. L^{-1}).

Wild-oat – fenoxaprop-P-ethyl (T/41)

De-transformed I_{50} values and resistance indices for the wild-oat biotypes LLUD and T/41 (target site resistance) are presented in Table 11 and fitted dose response curves shown as Figure 7 a-d. For both tests, there were significant differences between R (T/41) and S (LLUD) biotypes for both length and fresh weight of new shoot growth.

Table 11. Doses of fenoxaprop-P-ethyl required to reduce assessed growth parameters by 50 % (I_{50}) and resistance indices (RI) in wild-oat (T/41 v LLUD).

	Assessed growth parameter			
Growth stage	Fresh weight		Length	
	I_{50} [1]	RI	I_{50}	RI
2 – 3 tiller	R – 1.31 S – 0.02	65.5	R – 2.38 S – 0.09	26.4
Tiller test	R – 4.06 S – 0.07	58.0	R – 5.69 S – 0.36	15.8

[1] De-transformed, mg a.i./L^{-1}

The resistance indices obtained for fresh weight parameters (65.5 and 58) in both tests were far higher than the corresponding indices for length of new shoot growth (26.4 and 15.8). The seedling test at the 2-3 tiller growth stage yielded larger differences between biotypes than that of the tiller test with resistance indices being 65.5 (fresh weight) and 26.4 (length) in the seedling test and 58.0 (fresh weight) and 15.8 (length). I_{50} values were higher for the R biotype (T/41) in the tiller test than the seedling test, 4.06 and 1.31 mg a.i. L^{-1} respectively for the fresh weight parameter, and 5.69 and 2.38 mg a.i. L^{-1} respectively for the length parameter.

Discussion

The presented results show that, in all tests, with all herbicides and all species that resistant biotypes can be differentiated from their susceptible counterparts using the fresh weight of new shoot growth. Fresh weight gave the most consistent results due to it being a more accurate indicator of propagule re-growth. The fresh weight of new shoot growth is also a more reliable indicator of the propagules response to herbicide treatment than length of new shoot growth when assessing assays conducted with isoproturon and chlorotoluron.

The substituted phenylurea hebicides, isoproturon and chlorotoluron, are photosystem II (PS II) inhibiting herbicides and at the higher doses employed in these assays may cause chlorosis and necrosis of the new shoot growth. The resistance assays with black-grass and Italian rye-grass employing PS II inhibitors utilise the difference in tissue damage between R and S biotypes to discriminate resistance or susceptibility, measuring the length of new shoot growth dose not take into account the relative herbicide damage of the tissue being assessed. This is why, in a few cases, the length of new shoot growth parameter could not discriminate

between R and S biotypes when exposed to dose ranges of isoproturon, whereas the corresponding fresh weight could (Photo 1).

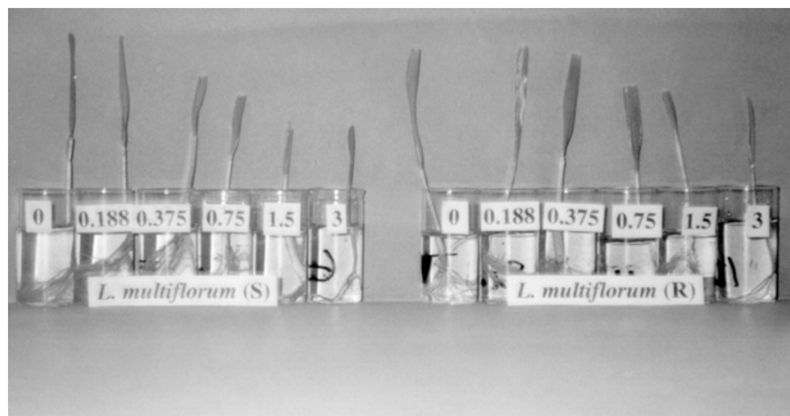


Photo 1. Seedling test with Italian rye-grass at the 2-3 tiller stage treated with a range of doses (0, 0.188, 0.375, 0.75, 1.5 and 3 mg a.i. L^{-1}) of isoproturon (Halja - S left, Clev - R right) seven days after treatment.

The results with black-grass found discrimination between R and S biotypes was far greater using cholotoluron than isoproturon in all growth stages, hence, it is suggested that this herbicide be used for testing for resistance to PS II inhibiting herbicides. The seedling tests with PS II inhibiting herbicides were assessed after seven days, however, greater distinction between biotypes may have been achieved if the assays were run for longer.

Fenoxaprop-P-ethyl is an aryloxyphenoxypropionic ACCase inhibiting herbicide and, over the dose-ranges employed in these assays, the herbicide damage manifests itself as growth inhibition as opposed to tissue damage (which would occur if higher doses were used). This is why, in general, I_{50} values were similar for both length and fresh weight of new shoot growth and that both parameters were equally good at determining herbicide resistance (Photo 2).

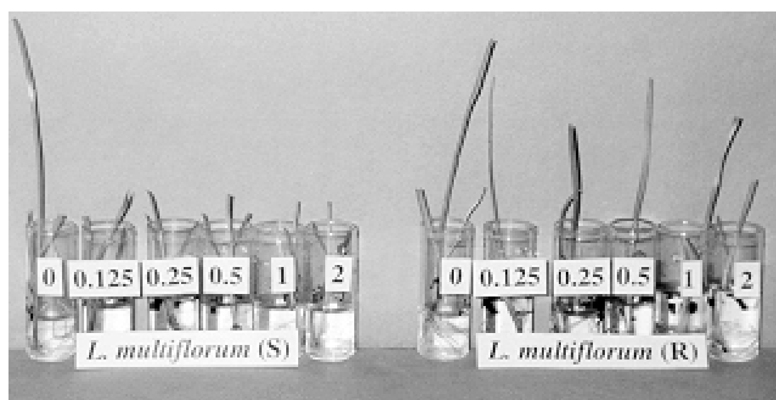


Photo 2. Seedling test with Italian rye-grass at the 2-3 leaf stage treated with a range of doses (0, 0.125 0.25, 0.5, 1 and 2 mg a.i. L^{-1}) of fenoxaprop-P-ethyl (Halja - R left, Clev - S right) seven days after treatment.

The study concentrated on detecting ACCase resistance in those biotypes that are resistant via enhanced metabolism. Enhanced metabolism based resistance to ACCase inhibiting herbicides is only partial, unlike target site resistance (insensitive ACCase), which is absolute. The results show that distinction between enhanced metabolism based resistant and susceptible biotypes was apparent in seedling tests conducted on black-grass and Italian rye-grass. Thus, it is assumed that the dose ranges used to used to discriminate between enhanced metabolism based ACCase resistant biotypes and susceptible biotypes will also detect target site resistance. This is shown in the case of wild-oat where the dose range employed to detect the enhanced metabolism based resistant biotype T/11 (0, 0.024, 0.074, 0.222, 0.666 and 2 mg a.i. L^{-1}) was far lower than that used for the detection of the target site resistant biotype T/41 (0, 0.25, 1.01, 43.06, 16.25 and 65 mg a.i. L^{-1}) .

HGCA funded a 10 week summer bursary project which compared the effects of alternative ACCase inhibiting herbicides on the outcome of herbicide resistance bioassays (Appendix A). Studies were also conducted on the acetolactate synthase (ALS) inhibiting herbicide flupyrsulfuron-methyl formulated as Lexus 50 DF (50 % w/w), however, consistent dose-responses or differentiation between S and R biotypes could not be achieved. Similar differentiation problems were encountered with flupyrsulfuron in Petri-dish seed assays (Stephen Moss *pers. comm.*).

Assays were conducted on seedlings from the 2-3 leaf stage to the 2-3 tiller stage, after the 2-3 tiller stage, tillers in all test species begin to root. Once rooted, tillers can be carefully teased

from the mainstem and used as seedlings. It is unlikely that the seedling test at the 2-3 leaf stage will be of much practical use as this will be the stage when herbicide is most likely to be applied. By the time a weed population is noticed to have survived a herbicide treatment (the earliest a population can be suspected resistant), the seedling test at the 4 leaf – 1 tiller growth stage would be youngest growth stage on which an assay would be conducted.

These dose-response assays have shown the ability to discriminate between S and R biotypes over a range of herbicide doses, even where the resistance is partial. A draft test protocol is attached as Appendix B. These experiments were all conducted on glasshouse grown plants,
the verification assays was undertaken at IACR - Rothamsted where the techniques were verified on plants grown under field conditions. It is envisaged that information from the dose-response assays will lead to a **final test using a single discriminating dose to detect herbicide resistance in all three species (See Validation exercise, Section 6)**

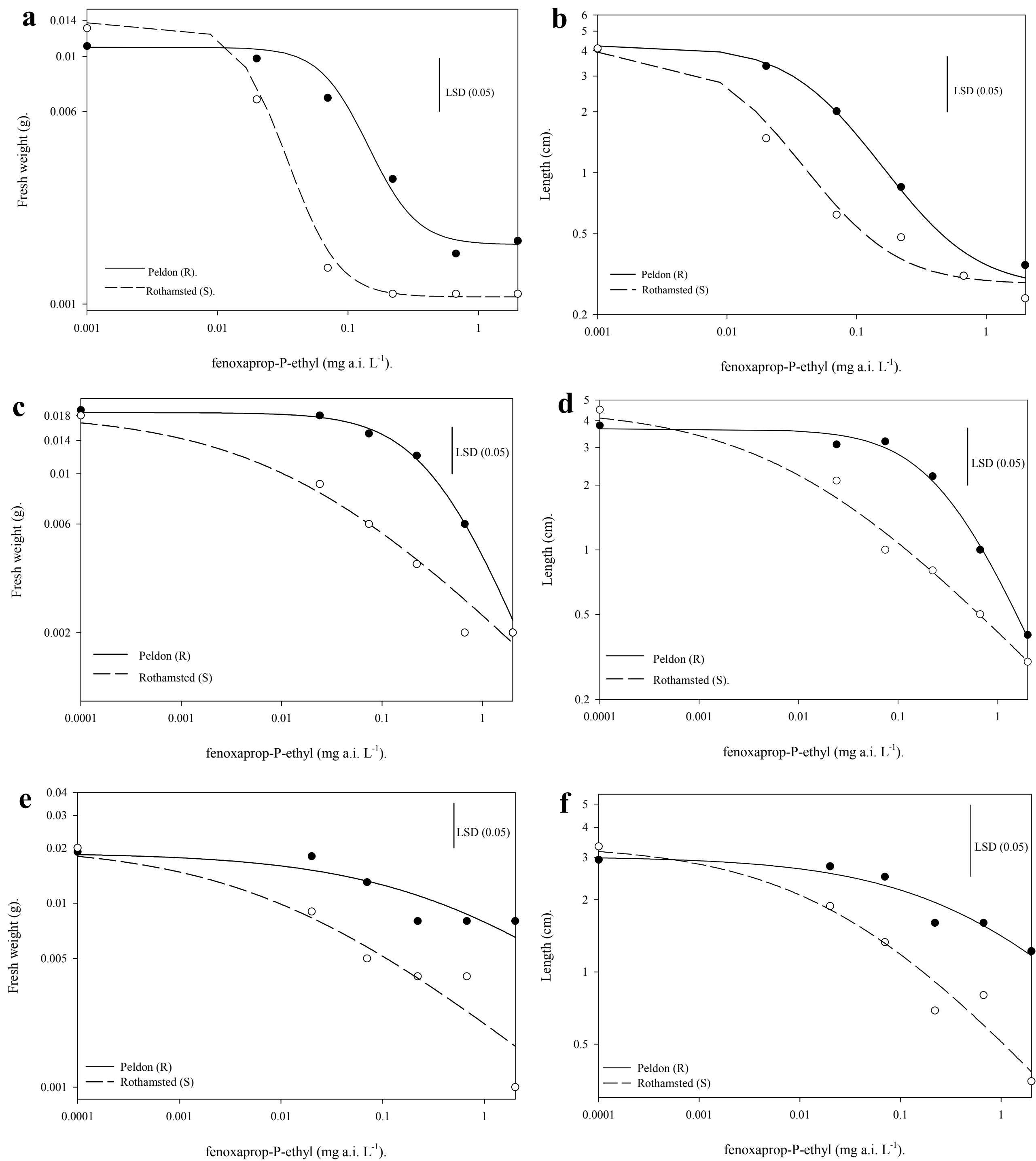


Figure 1 a-f. Fresh weights (a, c and e) and lengths (b, d and f) of trimmed propagules of black-grass treated with fenoxaprop-P-ethyl at various growth stages (1a and b – seedling test at 2-3 leaf stage, 1 c and d – seedling test at 2-3 tiller stage and 1 e and f – tiller test).

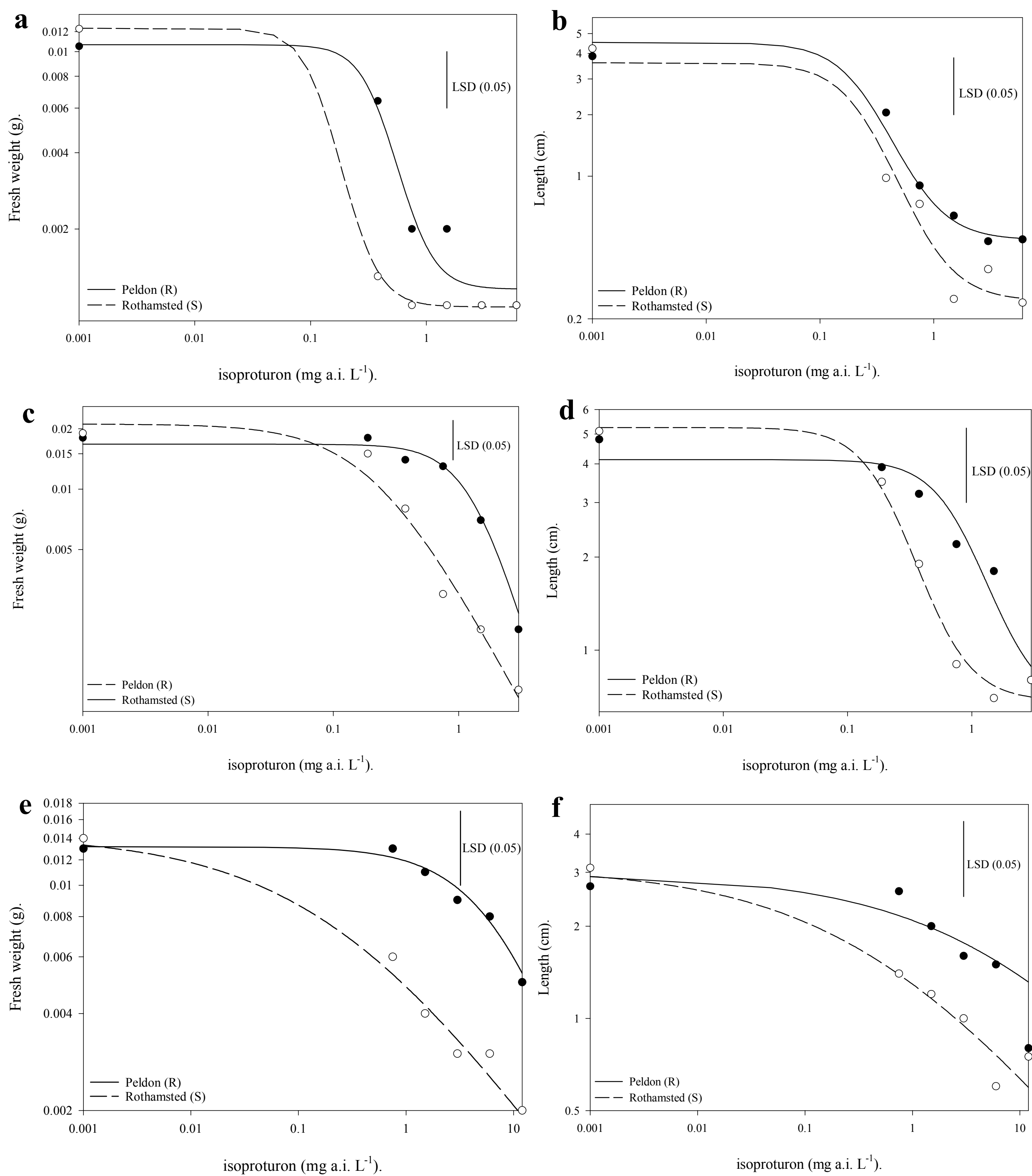


Figure 2 a – f. Fresh weights (a, c and e) and lengths (b, d and f) of new shoot growth of trimmed propagules of black-grass treated with isoproturon at various growth stages (2 a and b – seedling test at 2-3 leaf stage, 2 c and d – seedling test at the 2-3 tiller stage and 2 e and f – tiller test).

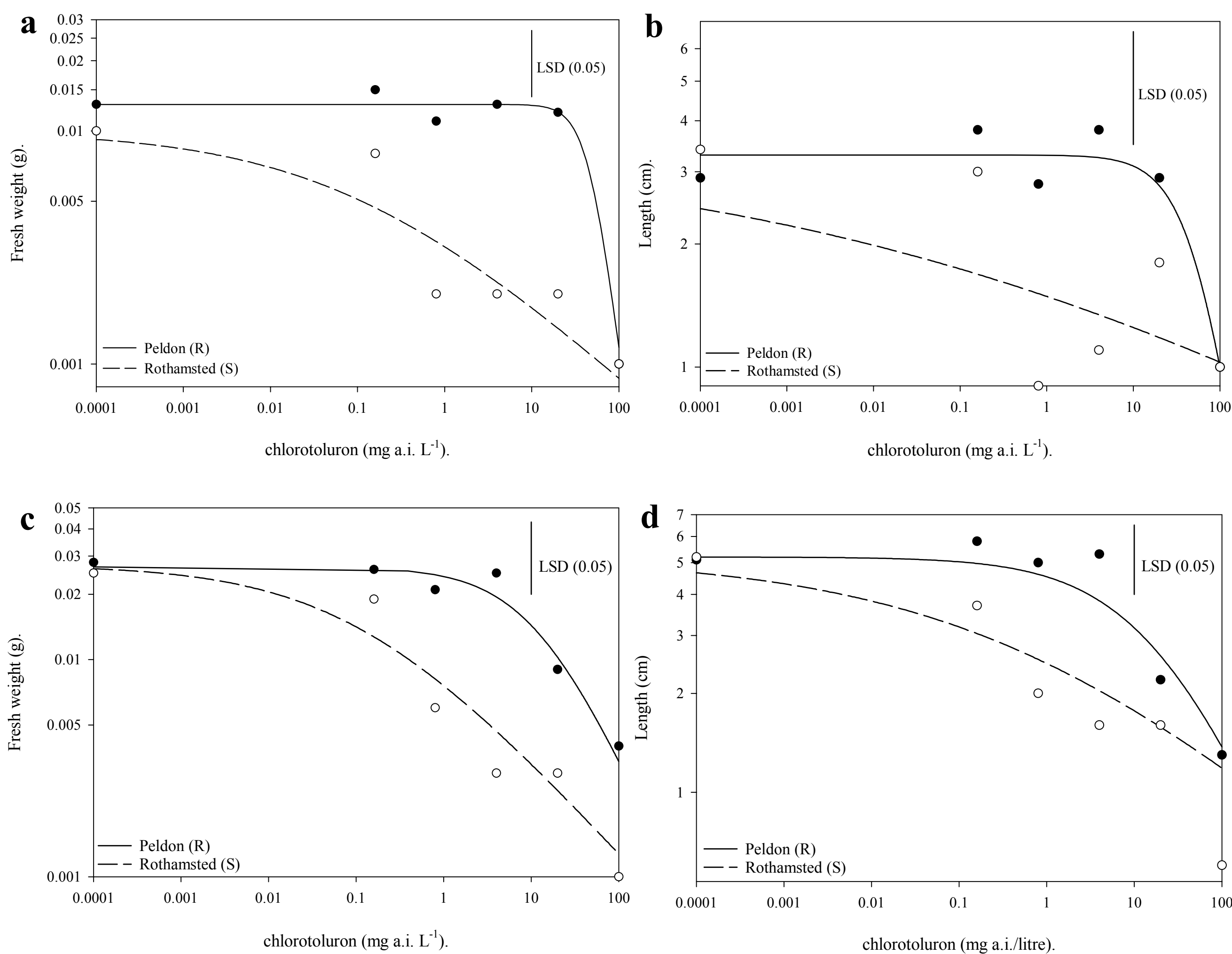
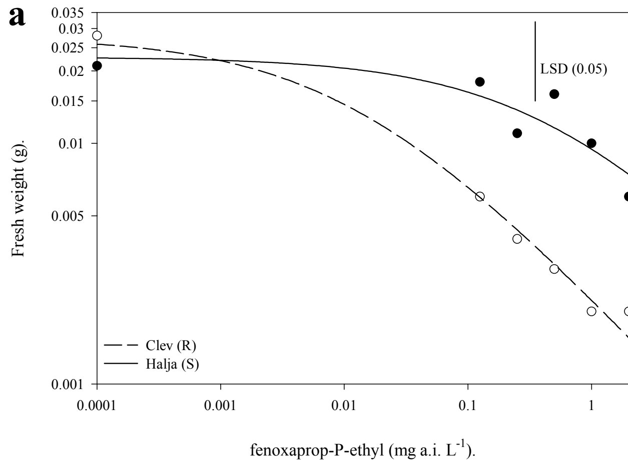


Figure 3 a-d. Fresh weights (a and c) and lengths (b and d) of new shoot growth of trimmed propagules of black-grass treated with chlorotoluron at various growth stages (a and b – seedling test 4 leaf – 1 tiller and c and d - tiller test).


a
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0.001
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
0.0001
0.001
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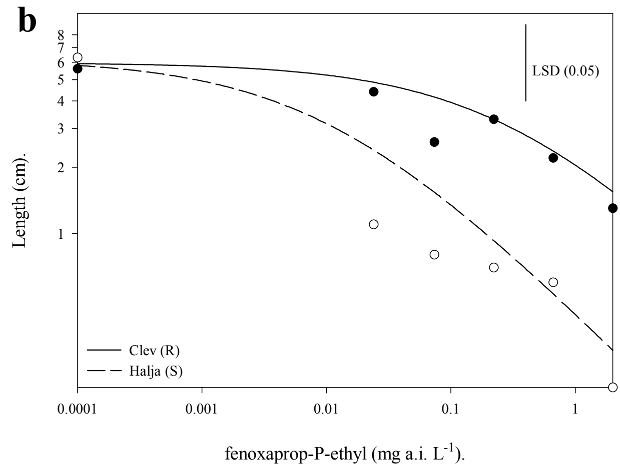
0.1
1
fenoxaprop-P-ethyl (mg a.i. L-1).


b
8
7
6
5
4
3
2
1
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
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0.001
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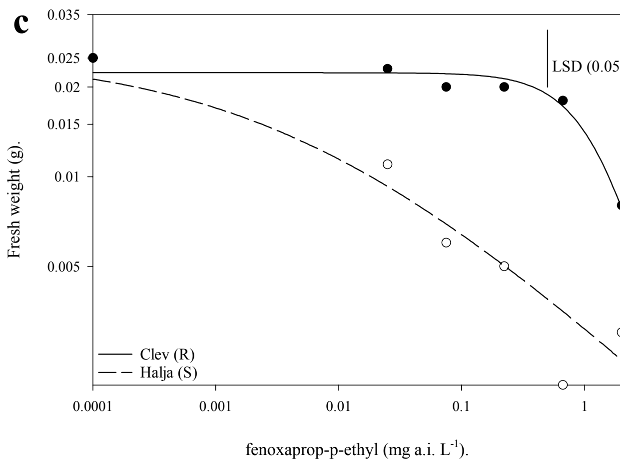
1
fenoxaprop-P-ethyl (mg a.i. L-1).


c
0.035
0.025
0.02
0.015
0.01
0.005
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
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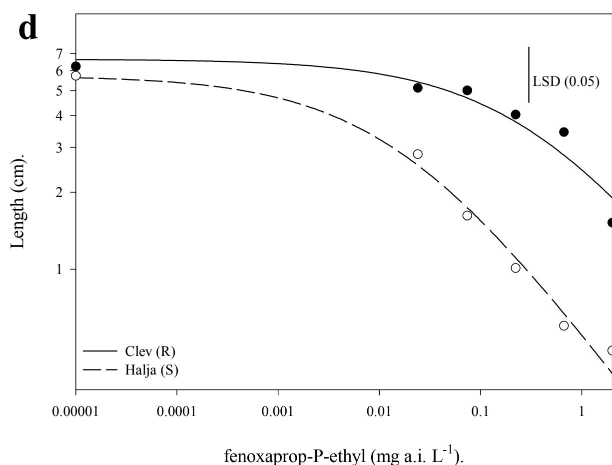
1
fenoxaprop-p-ethyl (mg a.i. L-1).


d
7
6
5
4
3
2
1
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
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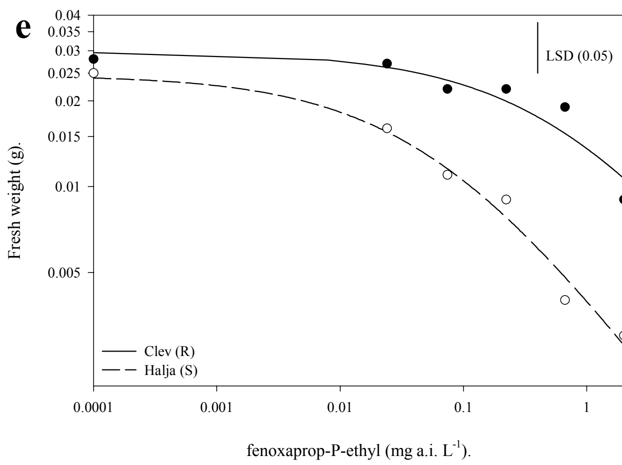
fenoxaprop-P-ethyl (mg a.i. L-1).


e
0.04
0.035
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0.02
0.015
0.01
0.005
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
0.0001
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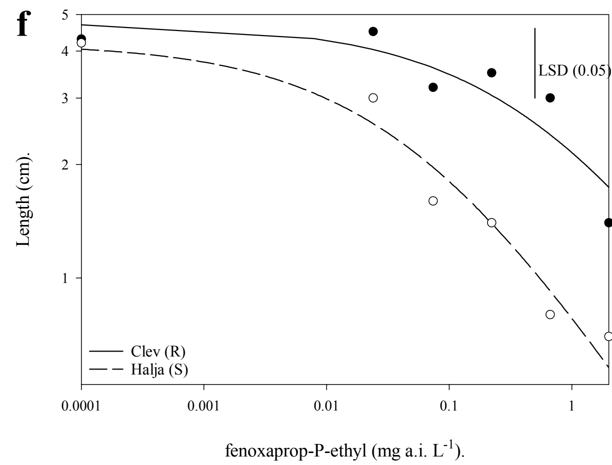
1
fenoxaprop-P-ethyl (mg a.i. L-1).


f
5
4
3
2
1
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
0.0001
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0.01
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1
fenoxaprop-P-ethyl (mg a.i. L-1).

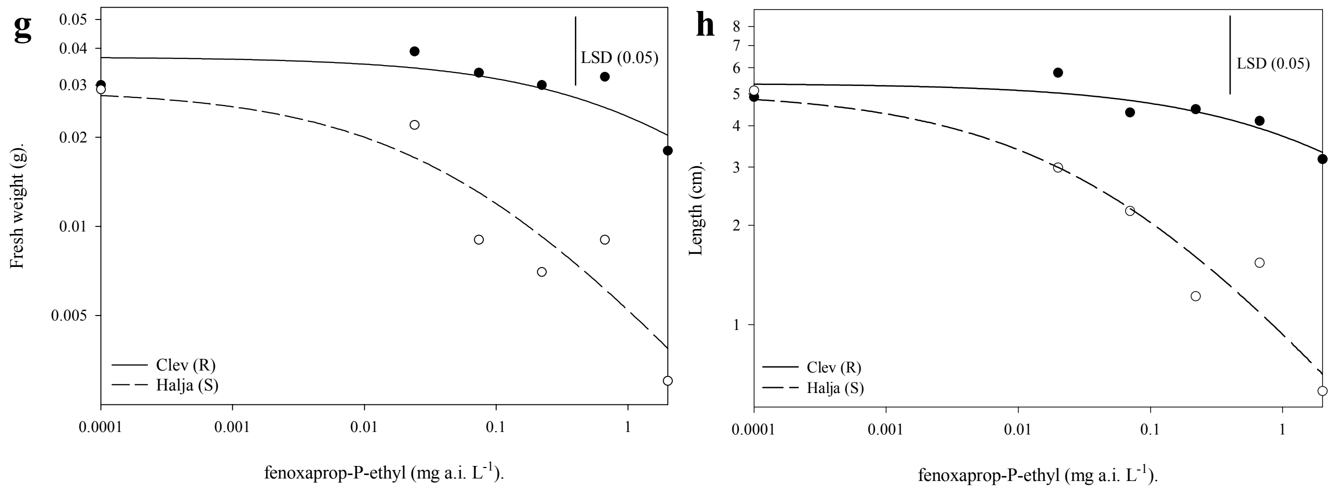
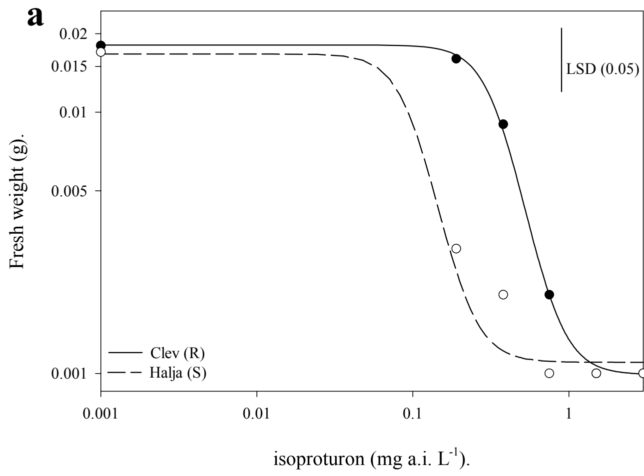
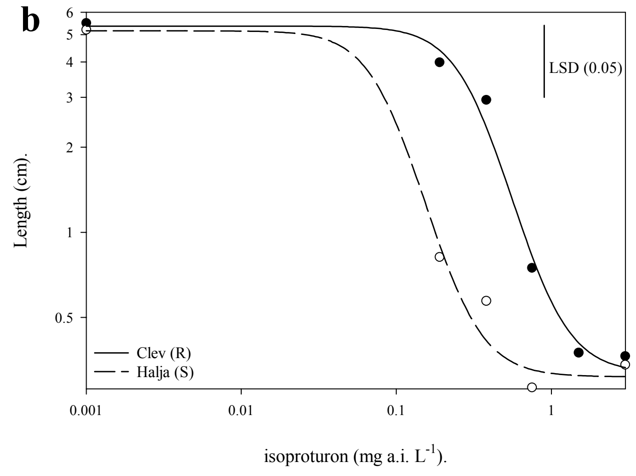


Figure 4 a – h. Fresh weights (a, c, e and g) and lengths (b, d, f and h) of new shoot growth of trimmed propagules of Italian rye-grass treated with fenoxaprop-P-ethyl at various growth stages (a and b – seedling test at the 2-3 leaf stage, c and d – seedling test at the 4 leaf – 1 tiller stage, e and f – seedling test at the 2-3 tiller stage and g and h – tiller test).


a
0.02
0.015
0.01
0.005
0.001
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
0.001
0.01
0.1
1
isoproturon (mg a.i. L-1).

b
6
5
4
3
2
1
0.5
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
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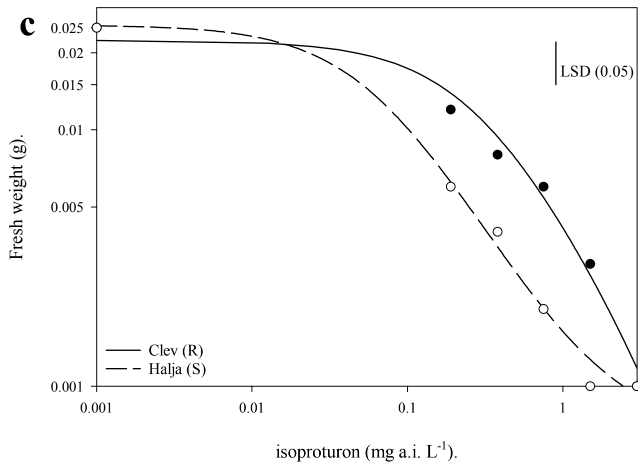
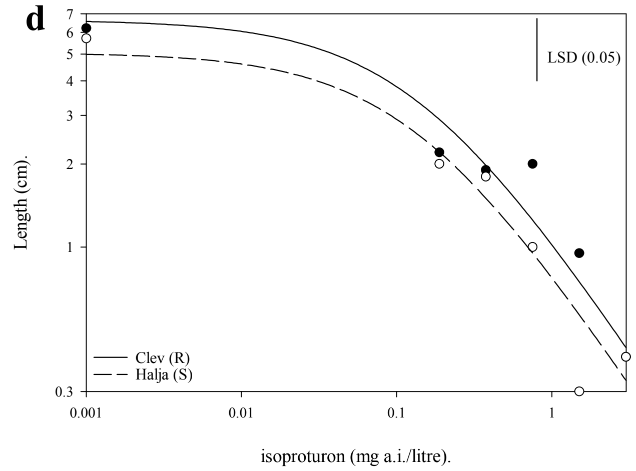
0.1
1
isoproturon (mg a.i. L-1).

c
0.025
0.02
0.015
0.01
0.005
0.001
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
0.001
0.01
0.1
1
isoproturon (mg a.i. L-1).

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6
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4
3
2
1
0.3
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
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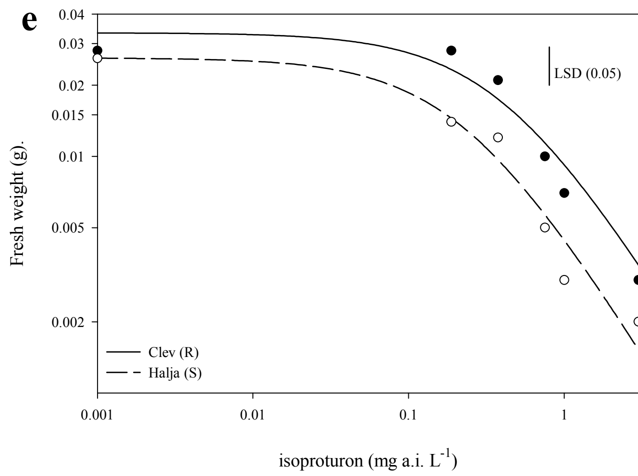
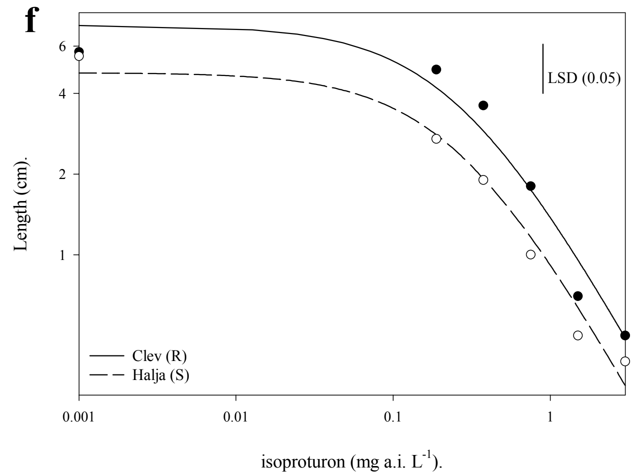
isoproturon (mg a.i./litre).

e
0.04
0.03
0.02
0.015
0.01
0.005
0.002
Fresh weight (g).
LSD (0.05)
Clev (R)
Halja (S)
0.001
0.01
0.1
1
isoproturon (mg a.i. L-1)

f
6
4
2
1
Length (cm).
LSD (0.05)
Clev (R)
Halja (S)
0.001
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isoproturon (mg a.i. L-1).

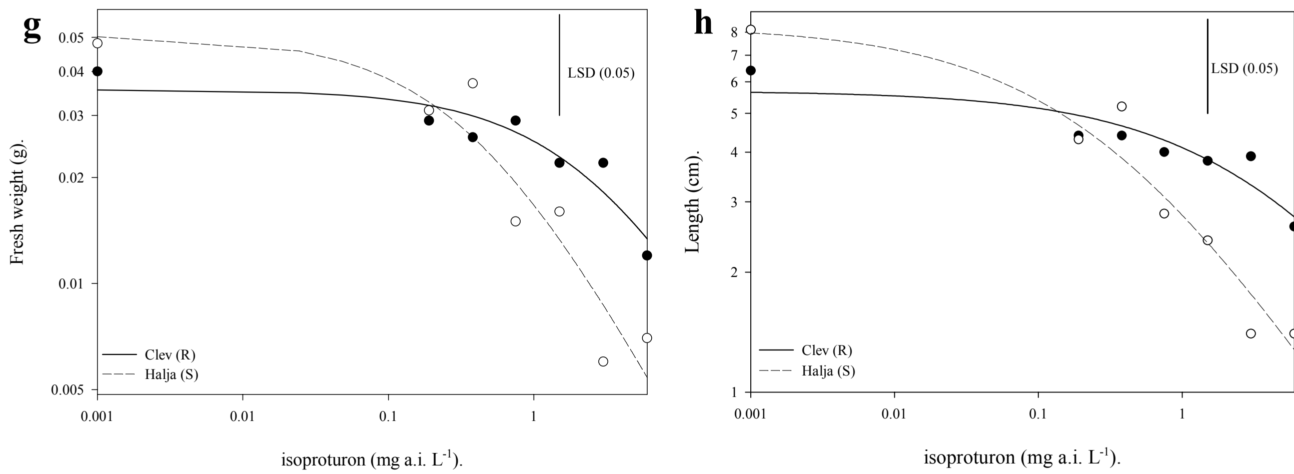


Figure 5 a-h. Fresh weights (a, c, e and g) and lengths (b, d, f and h) of new shoot growth of trimmed propagules of Italian rye-grass treated with isoproturon at various growth stages (a and b – seedling test at the2-3 leaf stage, c and d - seedling test at the 4 leaf – 1 tiller stage, e and f – seedling test at the 2-3 tiller stage and g and h – tiller test).

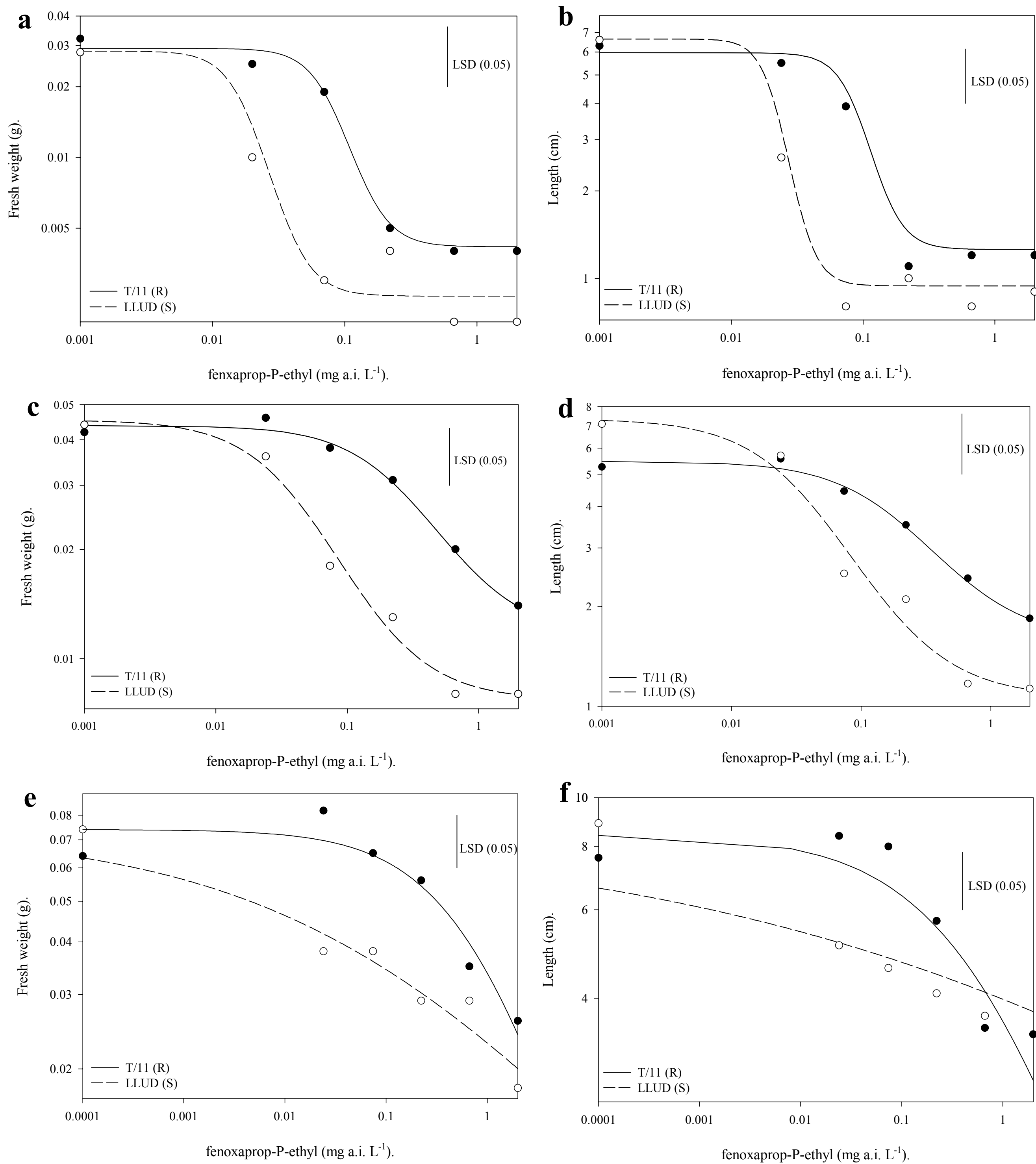


Figure 6 a-f. Fresh weights (a, c and e) and lengths (b, d and f) of new shoot growth of trimmed seedlings of wild-oat treated with fenoxaprop-P-ethyl at various growth stages (6 a and b – 2-3 leaf stage, 6 c and d – 4 leaf – 1 tiller stage and 6 e and f – 2-3 tiller growth stage.

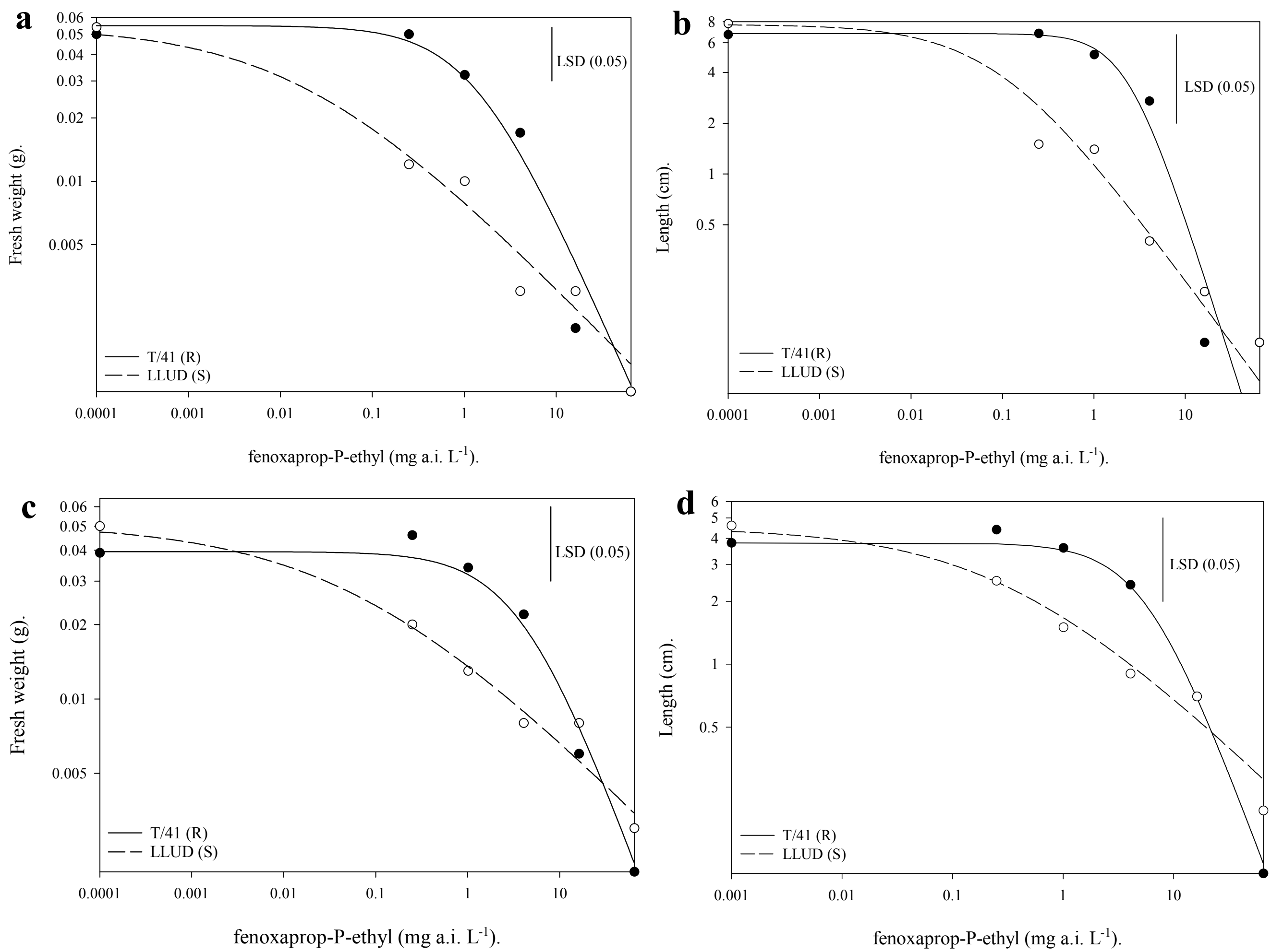


Figure 7 a-d. Fresh weight (a and c) and length (b and d) of new shoot growth of trimmed propagules of wild-oat treated with fenoxaprop-P-ethyl at various growth stages (7 a and b – seedling test at the 2-3 tiller stage and 7 c and d – tiller test).

3. STEM NODE TEST

The tiller test is suitable for use in herbicide resistance bioassays so long as enough tillers are available at a suitable growth stage. Once the first node is detectable on the tillers, they are unsuitable for use. Hence, a new technique is required for the detection of herbicide resistance once stem elongation has begun. A method was employed by Kim *et al*., (2000) which utilised cut stem node fragments of susceptible and resistant biotypes of *Echinochloa colona. E. colona* may be vegetatively propagated by production of new roots and shoots at the nodes when it is in a stage of prostrate growth (Holm *et al*., 1977). Kim *et al*., (2000) found that these stem node fragments readily produced adventitious roots after 48 hours with the node immersed in water, stem node segments of R and S *E. colona* were then immersed in herbicide solution and fresh weight and length of new shoot growth after 10 days were used to discriminate between R and S biotypes.

It was assumed that this method would also work in black-grass, Italian rye-grass and wild-oat. Initial studies with wild-oat found that no viable (defined to be those with new shoot and root growth over 5 cm in length) propagules were formed even after three weeks immersion in deionised water. Hence, other methods were employed including the use of the growth regulators and nutrient solution to encourage new root and shoot formation but there was no increase in viable propagule formation.

In order to form vegetative propagules that would be suitable for use in bioassays for the detection of herbicide resistance an alternative method was tried. Wild-oat at the ear-emergence/flowering stage were re-trimmed to soil level in the hope that the resultant new growth could be utilised in resistance assays. After 36 days of 're-growth' no viable propagules could be obtained, furthermore, the new growth had detectable nodes and ear emergence had begun in some 'tillers'. It was postulated that the onset of flowering had been induced by a long photoperiod (the original plants had been re-trimmed in late May) hence, wild-oat plants at the ear-emergence/flowering stage were again re-trimmed to soil level but transferred to a controlled environment (CE) facility set to a 12 ^{o}C, 10 hour day, 8 ^{o}C, 14 hour night in order to attempt to negate the onset of flowering. Reduced photoperiod and temperature had no effect, after 34 days of re-growth stem elongation had occurred in all 'tillers' and ear-emergence in some (Photo 3 a and b).

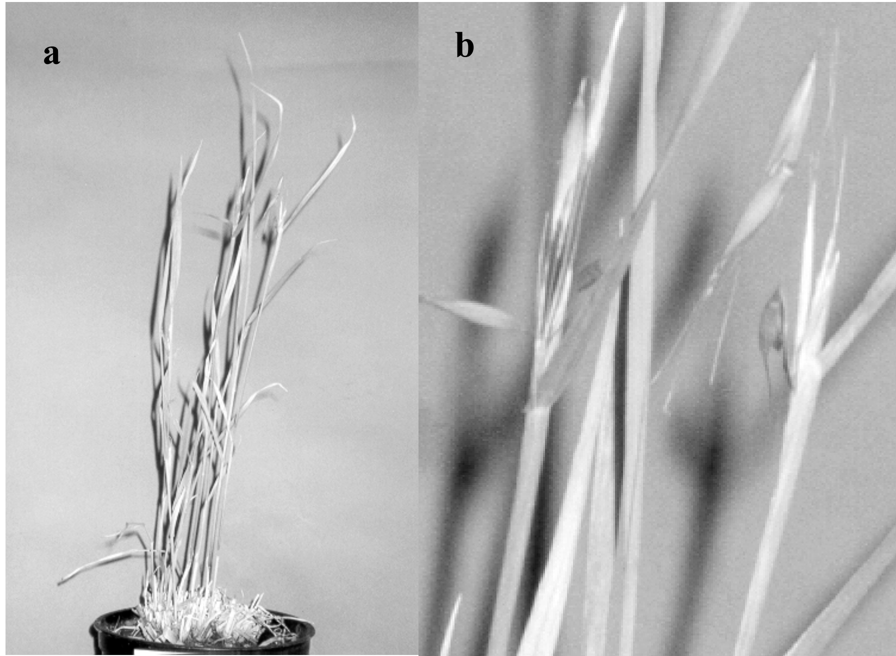


Photo 3 a and b. Re-growth of wild-oat (LLUD) that had been trimmed to ground level 34 days after trimming where a - showing stem elongation of 'tillers' and b – ear-emergence.

Black-grass and Italian rye-grass stem node segments produced new shoot and root growth far more readily than wild-oat. Segments of black-grass and Italian rye-grass that were cut from nodes 1, 2 or 3 above ground produced viable propagules (having 5 cm or more new root and shoot growth) in 10 % of black-grass and 20 % of Italian rye-grass segments after 17 days soaked in water. When these R and S stem node propagules were trimmed to 5 cm shoot and 5 cm root and exposed to a range of herbicide doses the results were unreliable. There was large variability within doses, with many propagules not growing at all, even in the controls.

Due to the low yield of stem node propagules suitable for bioevaluation and their extreme variability when assayed, resistance would be more reliably detected using assays conducted on seeds of suspected R populations, such as the Rothamsted Rapid Resistance Test (Moss, 1999). By the time 2 or 3 nodes are detectable on a weed species it is too late to take remedial action, thus there is no penalty in waiting for the results of seed assays.

4. ROBUSTNESS OF ASSAYS UNDER CONTRASTING TEMPERATURE, HUMIDITY AND LIGHT CONDITIONS

Single dose assays were conducted with all species using representative herbicide modes of action over contrasting temperature, humidity and light conditions. These were conducted with young seedlings (2-3 leaf) only, on the premise that they would be the propagules which would be most affected by change in environmental conditions.

In order to assess the importance of individual environmental factors on the outcome of assays for the detection of herbicide resistance controlled environment facilities were employed to vary one parameter (either light, temperature or humidity) between CE cabinets whilst keeping the remaining factors constant. Thus, experiments were duplicated, with one conducted at one parameter extreme and the other conducted at the contrasting extreme.

Materials and method

Plant material

All seedlings were sown as mentioned in section 2 (dose-response assays). Seedlings were harvested at the 2-3 leaf stage of growth and treated as for the seedling test in section 2. The biotypes used were as for dose-response assays but only one R biotype was used for wild-oat (T/11). Trimmed seedlings were placed in 10 ml glass vials containing 8 ml herbicide solution (see table 12). There were ten replications per biotype per dose per environmental variable. Experiments were fully randomised.

Herbicides and doses

These assays were conducted on single doses derived from dose-response experiments. These doses were chosen to discriminating between R and S biotypes and are shown in table 12.

Table 12. Species, herbicides and dose combinations employed to test the robustness of the assays.

Species	Herbicide	Doses (mg a.i. L^{-1})
Black-grass	fenoxaprop-P-ethyl [1]	0, 0.07
	isoproturon [2]	0, 0.2
Italian rye-grass	fenoxaprop-P-ethyl	0, 0.07
	Isoproturon	0, 0.2
wild-oat [3]	fenoxaprop-P-ethyl	0, 0.07

[1] formulated as 'Cheetah S'.
[2] formulated as 'Stress'.
[3] biotypes LLUD (S) and T/11 (enhanced metabolism resistance).

Assay conditions

Table 13 shows the environmental factors which were varied (variable parameter) together with the contrasting levels at which they were tested whilst the final column (constants) lists the factors and levels at which they were held.

Table 13. Controlled environment conditions employed for robustness assays.

Variable parameter	Contrasting levels	Constants [1].
Light	200 µmol m^{-2} s^{-1} vs	Temperature (17 °C d, 10 °C n)
	100 µmol m^{-2} s^{-1}	Humidity (75 % RH)
Temperature	17 °C day, 10 °C night vs	Light (200 µmol m^{-2} s^{-1})
	10 °C day, 5 °C night	Humidity (75 % RH) [2]
Humidity	90 % RH vs 50 % RH	Temperature (17 °C d, 10 °C n)
		Light (200 µmol m^{-2} s^{-1})

[1] 16 hour day, 8 hour night.
[2] humidity adjusted at low temperature regime to take into account vapour pressure deficit.

Light and humidity levels were enforced due to CE cabinet constraints. Temperature regimes were selected following preliminary studies (results not presented), which identified 17 °C / 10 °C as an optimum, assays were conducted at 25 °C / 15 °C and good discrimination between biotypes were achieved but these 'high' temperatures encouraged algal contamination which invalidated the results.

Assay assessment and analysis

Length and fresh weight of new shoot growth were assessed 7 days after treatment and results subject to $\log_e$ transformation prior to ANOVA in order to detect overall effects on the outcome of assays between contrasting conditions and differences between biotypes under individual conditions.

Results

Effect of light on the outcome of assays

Table 14. The effect of two different light levels on the fresh weight and length of new shoot growth of S/R biotypes of three weed species treated with a discriminating herbicide dose.

Species	Herbicide	Parameter	Significant differences between R/S biotypes at 200 µmol m^{-2} s^{-1}	Significant differences between R/S biotypes at 100 µmol m^{-2} s^{-1}	Significant light interaction on outcome of assay.
BG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N
	isoproturon	F wt	Y	Y	N
		Length	Y	Y	N
IRG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	Y
	isoproturon	F wt	Y	Y	Y
		Length	Y	Y	N
WO	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N

Y = Yes N = No

Table 14 and Figures 8 and 9 show for all three species there were significant differences between S and R biotypes at both light levels. The assays with Italian rye-grass treated with isoproturon showed that differences in responses existed between the two different light levels, the fresh weight of new shoot growth of the S seedlings at 100 µmol m^{-2} s^{-1} was significantly higher than at 200 µmol m^{-2} s^{-1} (Figure 8 d). However, at individual light levels there were significant ($LSD_{0.05}$) differences between R and S biotypes. There was also a significant light interaction on the outcome of assays at two light levels for the length of new shoot growth of Italian rye-grass treated with fenoxaprop-P. Again, significant differences between R and S biotypes existed at each individual light level.

Effect of temperature on the outcome of assays

Table 15 and Figures 10 and 11 show the effects of two different temperature regimes on the outcome of single dose assays for the detection of herbicide resistance in all three species.

Table 15. The effect of two different temperature regimes on the fresh weight and length of new shoot growth of S/R biotypes of three weed species treated with a discriminating herbicide dose.

Species	Herbicide	Parameter	Significant differences between R/S biotypes at 17 ^{o}C / 10 ^{o}C	Significant differences between R/S biotypes at 10 ^{o}C / 5 ^{o}C	Significant temp interaction on outcome of assay.
BG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N
	isoproturon	F wt	Y	Y	N
		Length	Y	Y	N
IRG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N
	isoproturon	F wt	Y	Y	N
		Length	Y	Y	N
WO	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N

Y = Yes N = No

They show that temperature had no significant overall effect on the outcome of single dose assays in any of the three test species for any of the herbicides. The absolute measurements were marginally higher at 17 ^{o}C / 10 ^{o}C than 15 ^{o}C / 5 ^{o}C (data not presented) but, for each species and each herbicide at both temperature regimes there were significant differences between R and S biotypes.

Effect of humidity on the outcome of assays

Table 16 and Figures 12 and 13 show the effects of two different humidity regimes on the outcome of single dose assays for the detection of herbicide resistance. For all species and all herbicides there were no significant humidity effects on the outcome of assays. Furthermore, all assays conducted resulted in significant differences ($LSD_{0.05}$) between S and R biotypes.

Table 16. The effect of two different humidity regimes on the fresh weight and length of new shoot growth of S/R biotypes of three weed species treated with a discriminating herbicide dose.

Species	Herbicide	Parameter	Significant differences between R/S biotypes at 90 % RH	Significant differences between R/S biotypes at 50 % RH	Significant humidity interaction on outcome of assay.
BG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N
	isoproturon	F wt	Y	Y	N
		Length	Y	Y	N
IRG	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N
	isoproturon	F wt	Y	Y	N
		Length	Y	Y	N
WO	fenoxaprop-P	F wt	Y	Y	N
		Length	Y	Y	N

Y = Yes N = No

Discussion

The robustness results show that for all the assays conducted significant differences were detected between R and S biotypes under every individual assay condition. Although in two cases, with Italian rye-grass, assays there were significant light effects, however, at each light level, discrimination between R and S biotypes were achieved (Table 14). It is recommended that assays are carried out at 17 ^{o}C, 16 hour day / 10 ^{o}C, 8 hour night because these were the conditions at which the herbicide doses were optimised.

The robustness studies showed that assays for the detection of herbicide resistance can be carried out under a variety of environmental conditions. If dose-response assays are carried out under conditions other than those reported in section 1 other parameters, such as length of time before assessment, may need to be altered to provide optimum results. For example, if an assay for detection of an ACCase inhibitor resistance is conducted at a low temperature regime, then 7 days may not be long enough. Alternatively, an assay for the detection of PS II resistance carried out under very high light levels may yield results sooner than the time periods reported for assays carried out at 200 µmol m^{-2} s^{-1}.

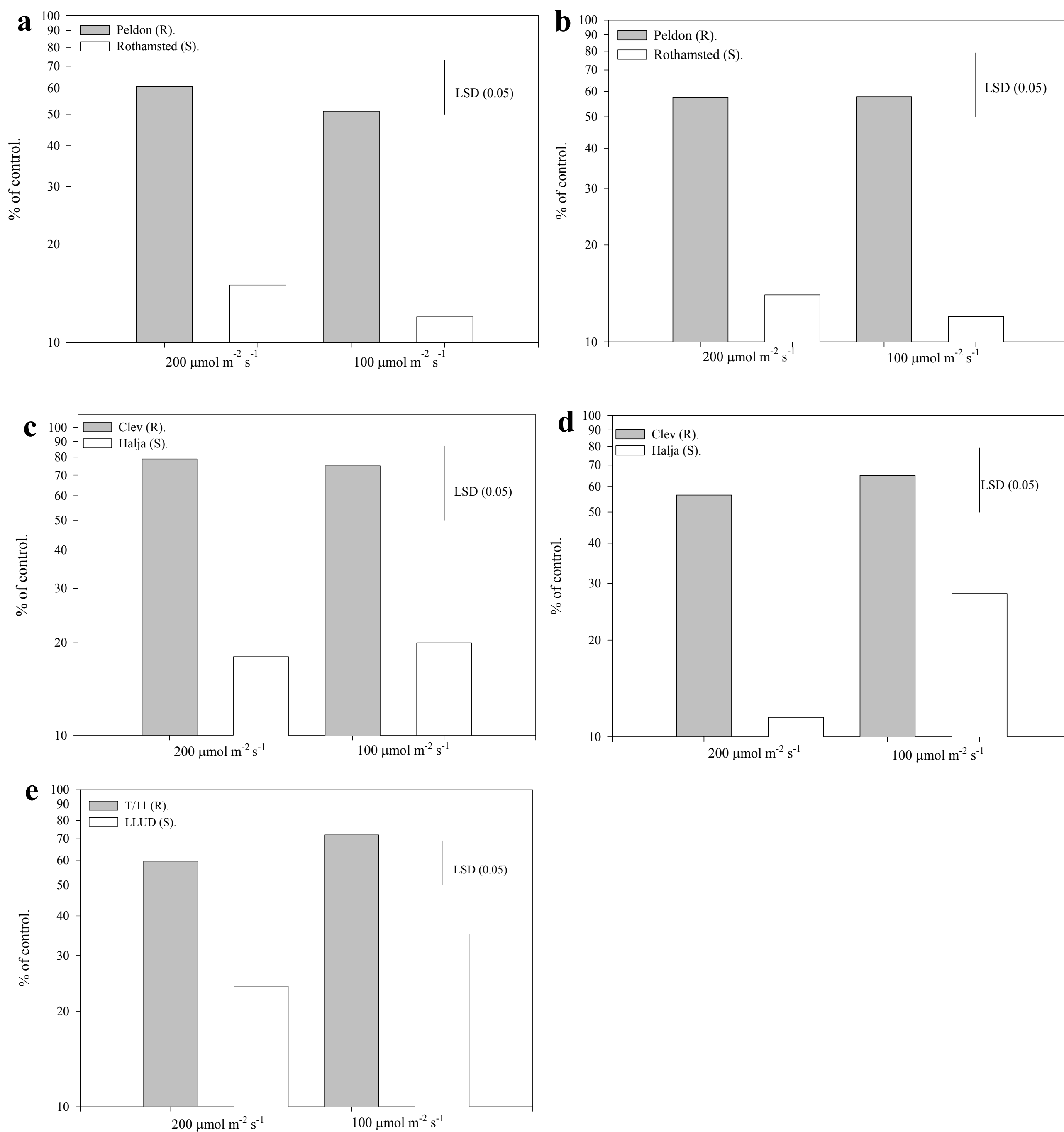


Figure 8 a-e. The effect of two different light levels on fresh weights expressed as a percentage of control of new shoot growth of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoproturon at 0.2 mg a.i. L^{-1} (b and d).

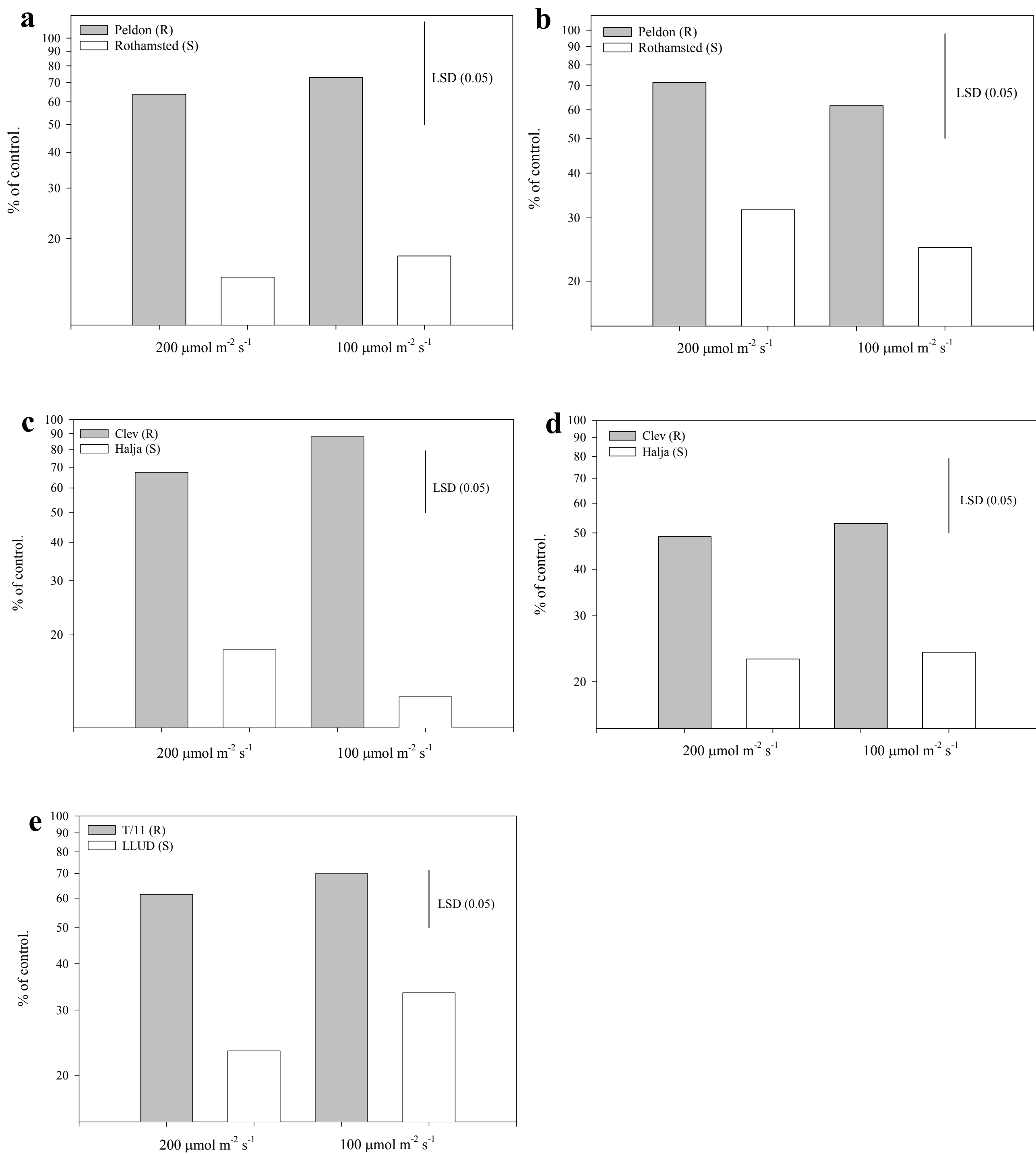


Figure 9 a-e. The effect of two different light levels on lengths expressed as a percentage of control of new shoot growth of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoproturon at 0.2 mg a.i. L^{-1} (b and d).

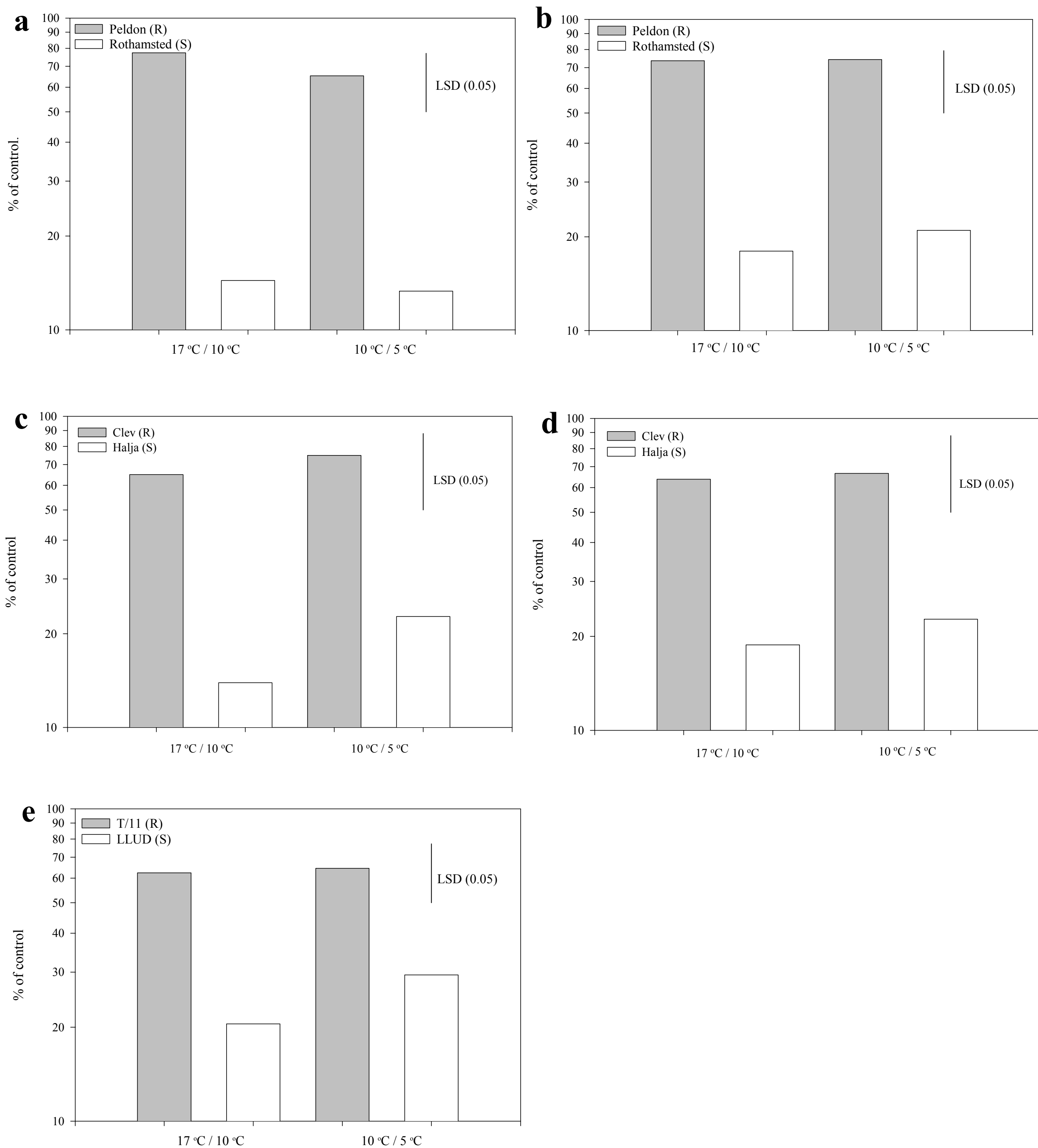


Figure 10 a-e. The effect of two different temperature regimes on fresh weights expressed as a percent of control of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoproturon at 0.2 mg a.i. L^{-1} (b and d).

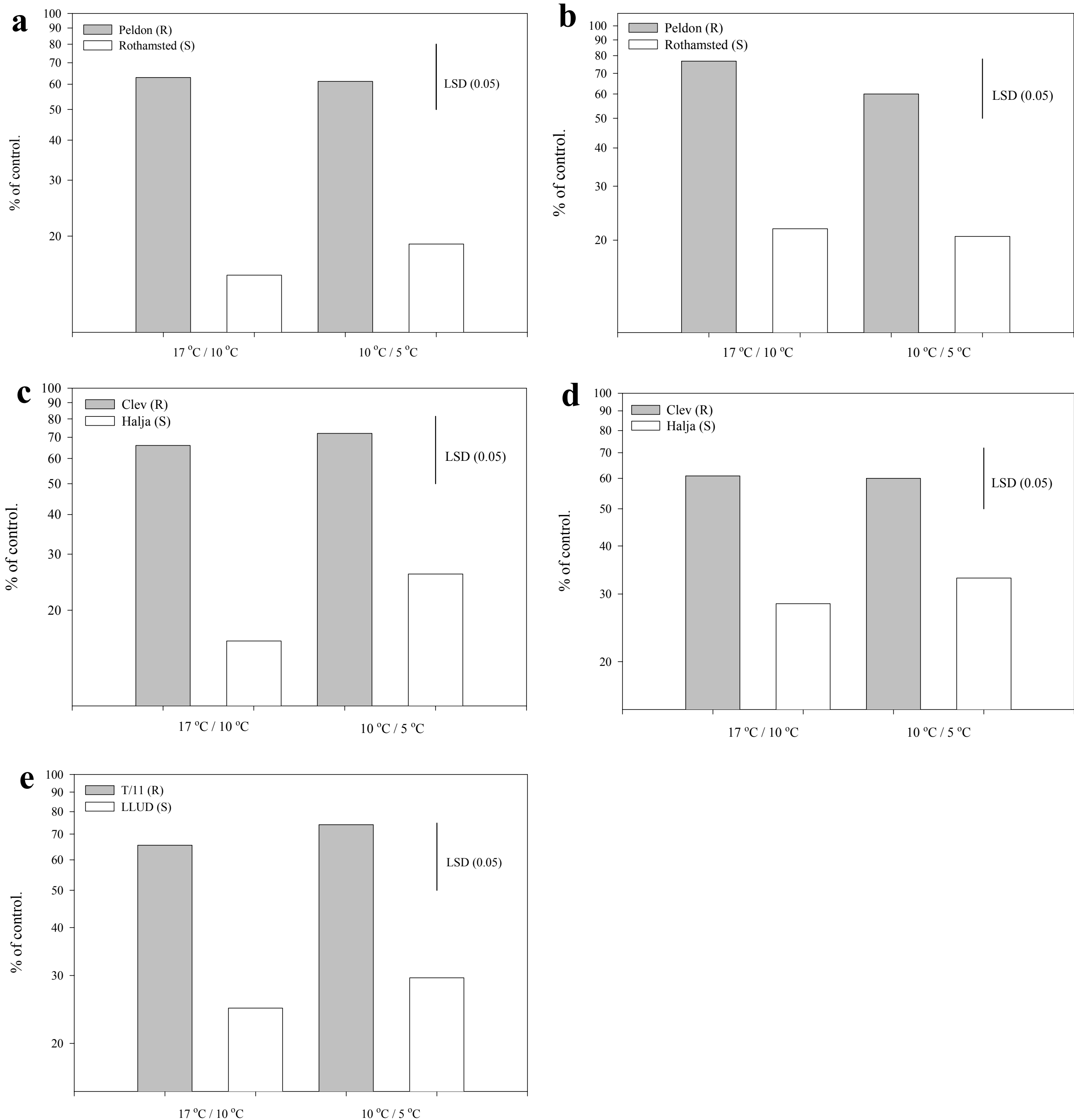


Figure 11 a-e. The effect of two different temperature regimes on lengths expressed as a percent of control of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoproturon at 0.2 mg a.i. L^{-1} (b and d).

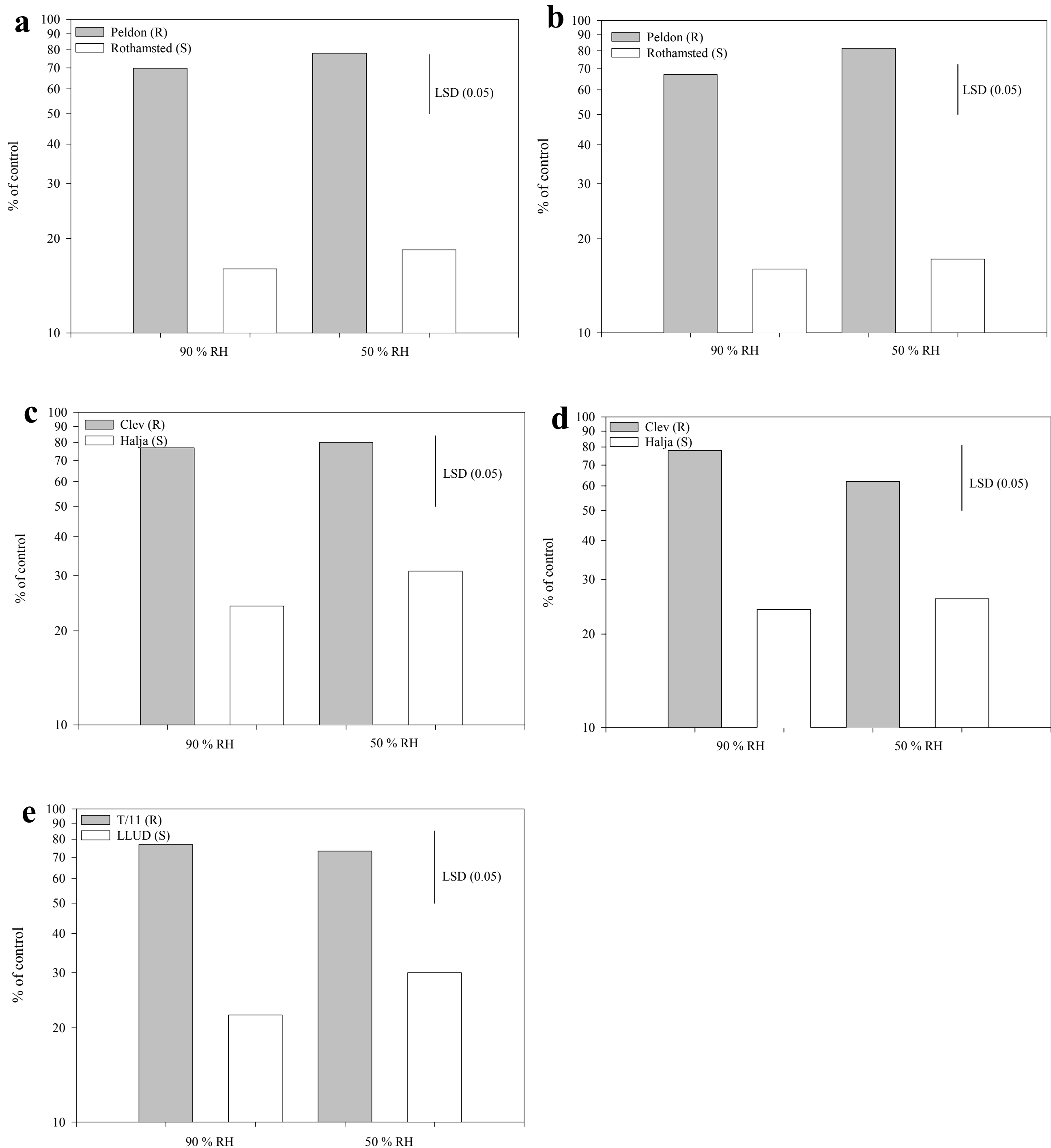


Figure 12 a-e. The effect of two different humidity regimes on fresh weights expressed as a percent of control of new shoot growth of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoprotuon at 0.2 mg a.i. L^{-1} (b and d)

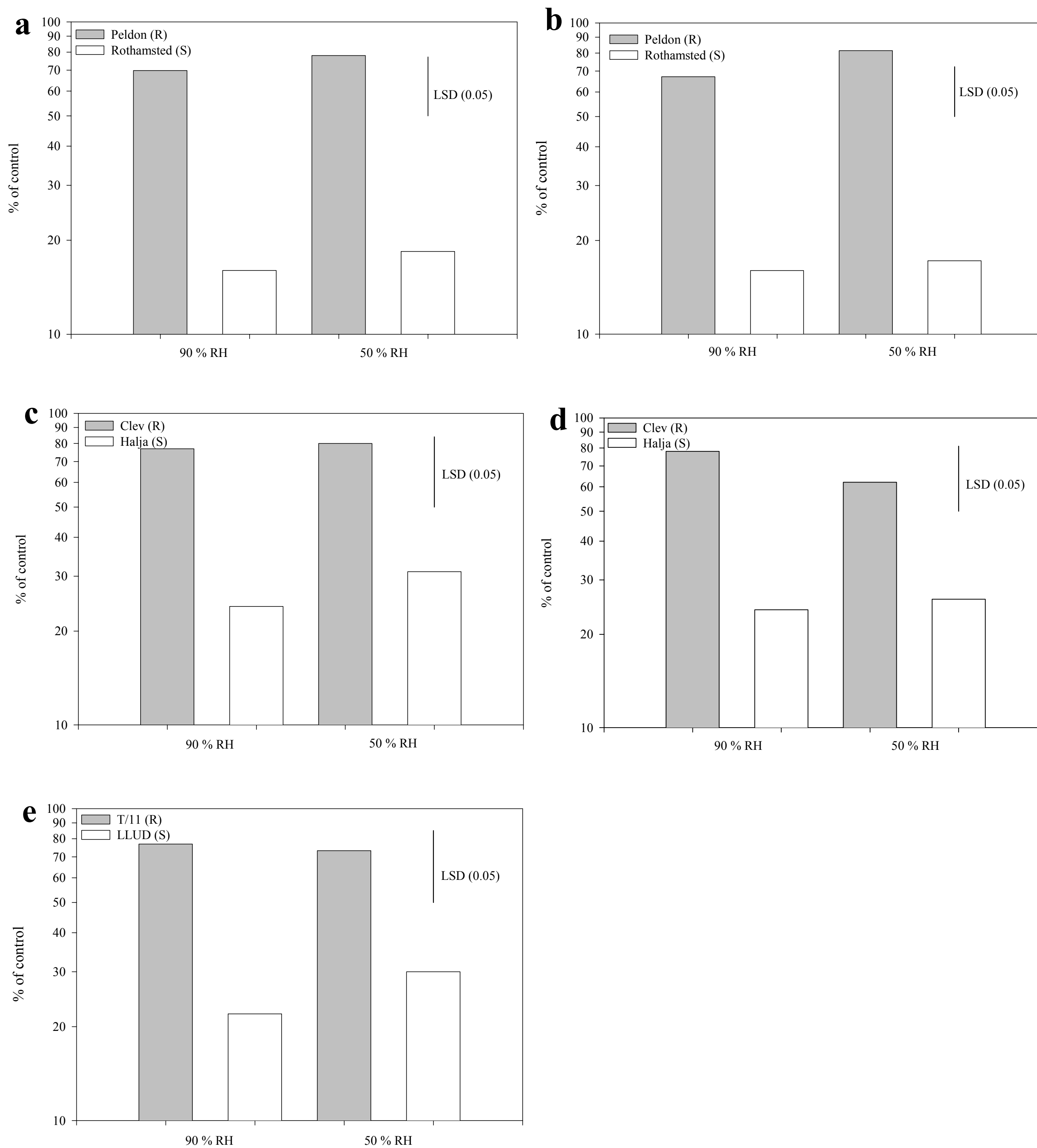


Figure 13 a-e. The effect of two different humidity regimes on length expressed as a percent of control of new shoot growth of trimmed seedlings at the 2-3 leaf growth stage of black-grass (a and b), Italian rye-grass (c and d) and wild-oat (e) treated with fenoxaprop-P-ethyl at 0.07 mg a.i. L^{-1} (a, c and e) or isoprotuon at 0.2 mg a.i. L^{-1} (b and d)

5. SAMPLE PACKAGING AND STORAGE

Under certain circumstances, there may be a need to store propagules for a certain period of time before a resistance assay can be carried out. This study assays seedlings and tillers of black-grass, Italian rye-grass and wild-oat in order to test propagule viability after storage.

Method and materials

Plant material

All plant material was sown and grown as mentioned in section 2. For this study, seedlings of all species were harvested at the 2-3 leaf stage, for tillers, black-grass and Italian rye-grass were harvested at the 6 – 10 tiller growth stage whilst wild-oats were at the 3 –5 tiller stage. After initial studies on tillers of all three species, it was found that an effective packaging method was to wrap the root system of trimmed and partially washed plants in 2 sheets of 'kim-wipe' blue role moistened with 30 ml deionised water and place the wrapped propagules in clear plastic bags and maintain at 1°C with light at 11 μmol m^{-2} s^{-1} for 16 hours a day (Photo 3).

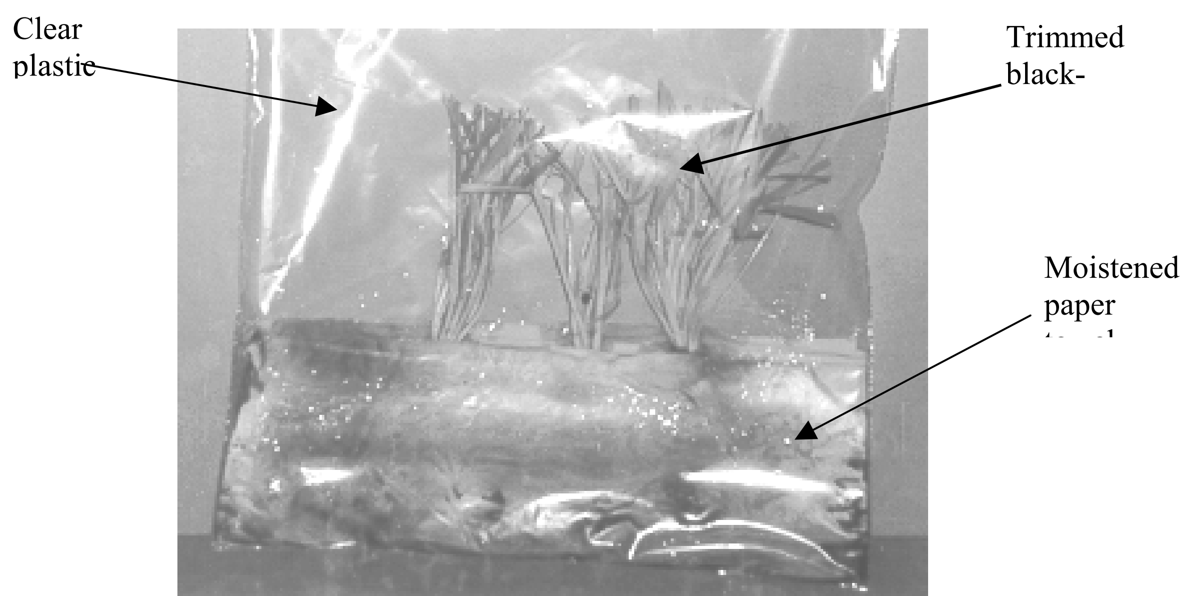


Photo 3. Trimmed black-grass at the 6 – 10 tiller stage of growth shown immediately prior to storage at 1°C.

Seedlings at the 2-3 leaf were left untrimmed, roots systems were carefully placed in folded paper towel and 30 were placed in every 25 x 30 cm bag, then 30 ml de-ionised water was

placed in every bag. The tillering plants of all species were trimmed to 10 cm shoot and 7 cm root prior to placement in paper towel and bags, there were 3 plants per bag.

After 31 days (seedlings) and 29 days (tillers) plants were removed from storage and assays carried out on the propagules to test their post-storage viability. Seedlings and tillers of all species were treated as in dose response assays. There were 10 replications per biotype per dose and experiments were fully randomised. Assays conducted in a CE facility operating at 17 ^{o}C 16 hour day, 10 ^{o}C 8 hour night.

Herbicide and doses

Table 17 shows the herbicide doses that the trimmed propagules were exposed to.

Table 17. Species, biotypes, tests and fenoxaprop-P-ethyl dose employed in storage assays.

Species	Biotypes	Test	fenoxaprop-P dose (mg a.i. L^{-1})
black-grass	Peldon, Rothamsted	ST, TT	0, 0.07, 0.14
Italian rye-grass	Clev, Halja	ST, TT	0, 0.07, 0.14
wild-oat	T/11, LLUD	ST, TT	0, 0.07

Assay assessment and analysis

Length and fresh weight of new shoot growth were assessed 7 days after treatment and results subject to log_e transformation prior to ANOVA in order to detect any differences between R and S biotypes response to herbicide treatment.

Results

Table 18 and Figures 14 and 15 show the effects of herbicide treatment on stored popagules of black-grass, Italian rye-grass and wild-oat. Differences between R and S biotypes response to fenoxaprop-P were apparent in both seedling tests at the 2-3 leaf stage and tiller tests for black-grass and Italian rye-grass for length and fresh weight of new shoot growth. Differences between R and S biotypes of wild-oat were only significant at the 2-3 leaf stage.

Table 18. The effect of treatment with fenoxaprop-P-ethyl on trimmed seedlings and tillers on three weed species that had been stored at 1 ^{o}C.

Species	Growth stage	Parameter	Significant differences between biotypes
Black-grass	2-3 leaf	F wt	Y
		Length	Y
	Tiller	F wt	Y
		Length	Y
Italian rye-grass	2-3 leaf	F wt	Y
		Length	Y
	Tiller	F wt	Y
		Length	Y
Wild-oat	2-3 leaf	F wt	Y
		Length	Y
	Tiller	F wt	N
		Length	N

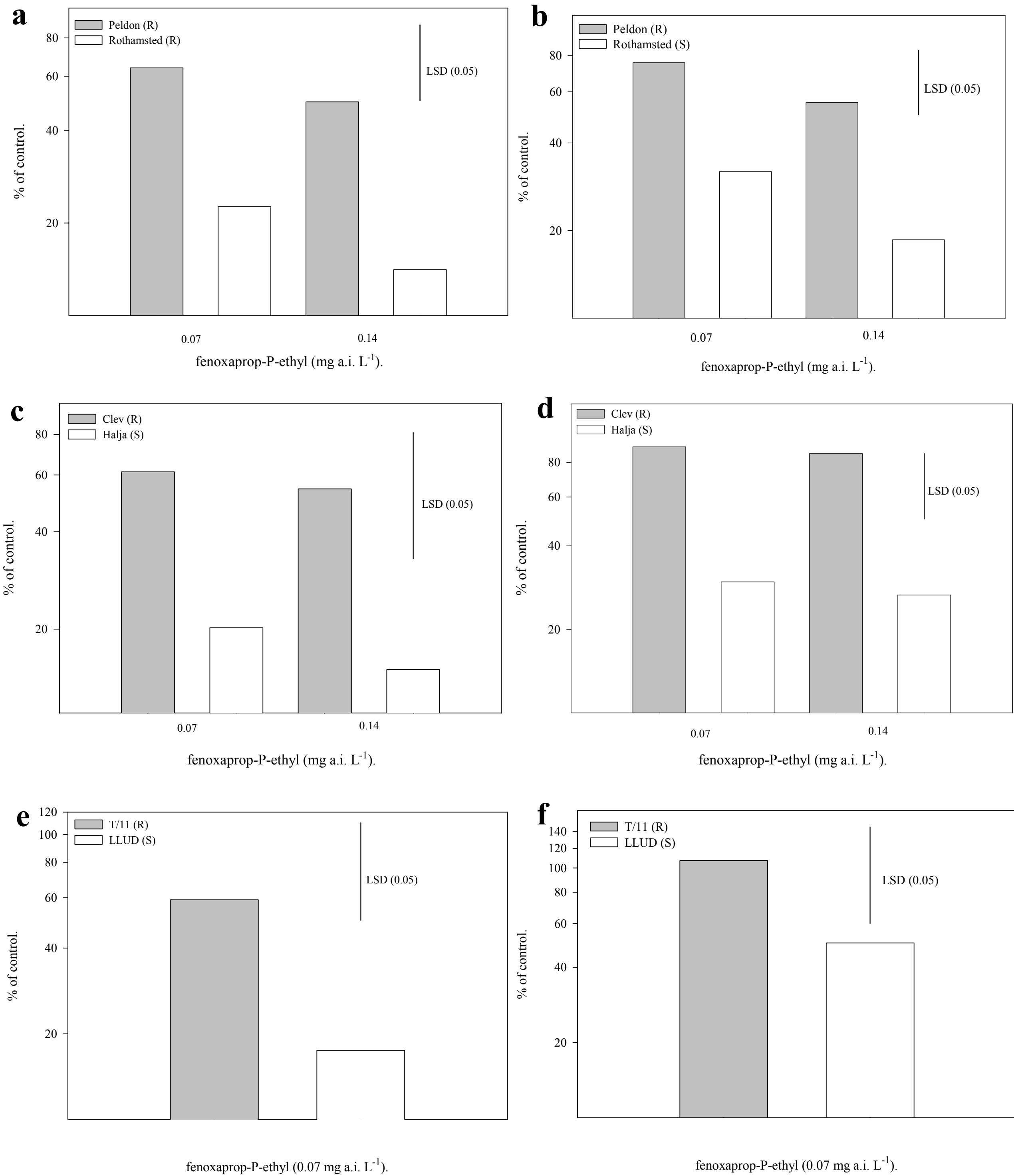
Y = Yes N = No

Discussion

The necessity to store propagules may arise, for example, if suspected R propagules have been collected from the field and no reference populations are available at a similar growth stage to assay them against. The suspected R population can then be stored whilst the reference populations reach the required growth stage.

These tests have demonstrated propagule viability of tillers and seedlings of all species after storage at 1°C for 29 and 31 days respectively. The tiller test carried out on wild-oat yielded no significant differences between R and S biotypes due to incorrect dose selection and not because of lack of propagule viability. Seedlings at the 2-3 leaf stage were chosen as they would be more susceptible to damage during storage. Tillers of all species were found to be viable even after 60 days in storage, although viability was only tested by shoot re-growth when propagules were placed in water (results not presented).

Initial studies with black-grass and Italian rye-grass at the 6-10 tiller stage and wild oats at the 3-5 tiller stage stored propagules at 5°C and 1°C and checked them for viability after 28 days. It was found that there was no effect of storage temperature on the outcome of assays,



however, the 1°C storage regime was preferred because there was less propagule growth and less water loss.

Figure 14 a-f. The effect of storage on fresh weights expressed as a percent of control of new shoot growth of trimmed seedlings at the 2-3 leaf stage (a - black-grass, c - Italian rye-grass and e - wild-oat) and trimmed tillers (b - black-grass, d - Italian rye-grass and e - wild-oat) treated with fenoxaprop-P-ethyl.

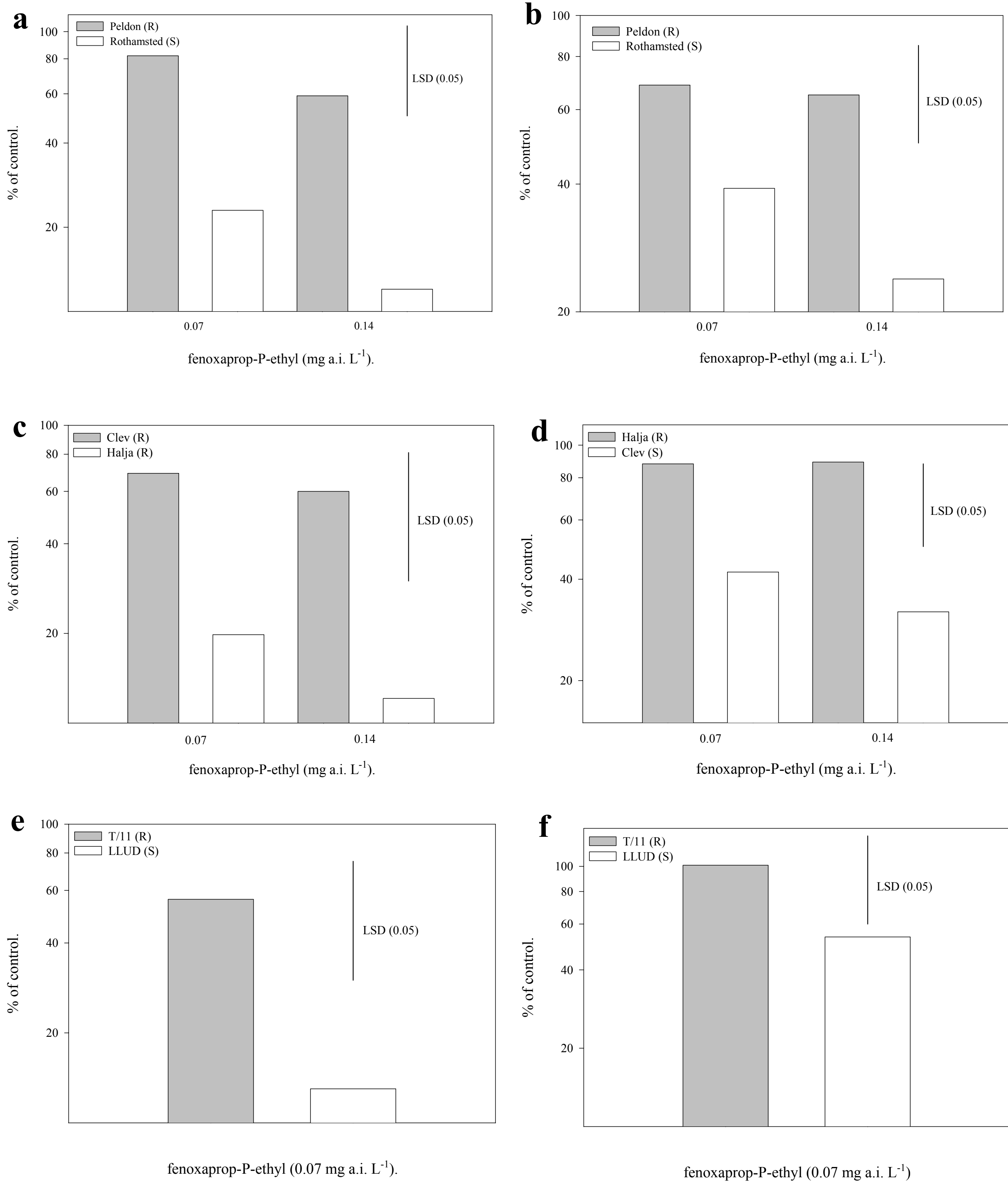


Figure 15 a-f. The effect of storage on lengths expressed as a percent of control of new shoot growth of trimmed seedlings at the 2-3 leaf stage (a - black-grass, c - Italian rye-grass and e - wild-oat) and trimmed tillers (b - black-grass, d - Italian rye-grass and f - wild-oat) treated with fenoxaprop-P-ethyl.

6. VALIDATION EXERCISE (IACR - ROTHAMSTED)

Objective

The aim was to investigate whether the resistance testing procedure, developed at Long Ashton using mainly glasshouse grown plants, could discriminate between susceptible (S) and resistant (R) black-grass plants collected from actual field situations. If the technique did work adequately, then the scope for simplifying it would be investigated with the aim of producing a simple, robust resistance testing protocol.

Methods

A series of experiments was conducted to refine the existing protocol for plants from the field. The experiments are described in logical order and the conclusions detailed.

Plant material (black-grass)

One resistant (**Woburn**) and one susceptible (**Rothamsted**) population of black-grass (*Alopecurus myosuroides*) were used in most studies. The susceptible plants were collected from a section of Broadbalk field at Rothamsted that has never received herbicide and the resistant plants from a field at Woburn, Bedfordshire where resistant black-grass had been identified in previous screening experiments. The Woburn population had a moderate level of target site and a low level of metabolism resistance. It was rated as 2* (RR) to chlorotoluron, 5* (RRR) to fenoxaprop and 3* (RR) to sethoxydim, (with an estimated 36% of plants with target site resistance) using the rating system described by Moss *et al.* (1999). This was a good population to use to test the robustness of the testing system as it is representative of many other resistant populations, and does not show atypically high levels of resistance. The intention had been to obtain plants from additional fields but the foot and mouth epidemic prevented this. However some other populations were used in individual experiments and these were obtained from outdoor containers at Rothamsted.

EXPERIMENT 1: DOSE RESPONSE ASSAY WITH FENOXAPROP

Materials and Methods

Black-grass plants (Rothamsted (= susceptible, S) and Woburn (= resistant, R) populations) at the 3 leaf to 2 tiller stage were dug up on 8 January 2001 with a trowel, with care taken to preserve their root structure. Soil was retained around the roots to better preserve the plant. The roots of these seedlings were then washed under a slow running tap before being trimmed

if necessary to 50 mm. The majority of the root lengths were between 30 and 40 mm. Any tillers were removed from the main stem and discarded at this stage. Plants were very variable in size even for the same growth stage. Mean shoot lengths of these plants were: Rothamsted 86 mm, Woburn 99 mm.

The initial fenoxaprop-P-ethyl test was started on 9 January 2001 using the formulated product 'Cheetah Super' (55g a.i. per L) with eight replicate plants per population at each of five doses (0.024, 0.074, 0.222, 0.666, 2.0 ppm), and 12 untreated control plants per population in demineralised water. These doses were prepared in a dilution series using demineralised water. A double dilution technique was used to obtain the highest concentration in order that readily available disposable syringes could be used, rather than expensive specialised micro-pipettes. 2.28ml of the commercial herbicide product was measured out with a 2.5 ml disposable syringe and mixed in 500 ml of water in a measuring cylinder. 4 ml of this solution was then measured out with a 5 ml syringe and made up to 500 ml with demineralised water in a second measuring cylinder. This produced a 2 ppm fenoxaprop solution. To produce lower concentrations, 167 ml of the 2 ppm solution was made up to 500 ml with demineralised water to produce a 0.666 ppm solution. This step was repeated to produce the 0.222, 0.074 and 0.024 ppm solutions. Solutions were decanted into 10 ml glass vials, with 8 vials per dose per population and 12 control vials per population. Vials were placed in polystyrene modules for ease of transport and storage, of the type normally used for growing seedlings.

Plants were trimmed across all their leaves with scissors at a point 1-2mm below the axil of their upper leaf and carefully placed in the appropriate vials. The plants were then transferred to a controlled environment (C.E.) cabinet set to provide a 14-hour (16°C) day and a 10-hour (10°C) night. After 24 hours in the C.E. cabinet the plants were removed, cut back to the point of the original cut (again, with scissors), and returned to the C.E. Trimming was repeated as shoots had elongated in the initial 24-hour phase irrespective of herbicide treatment.

Seven days after re-trimming, the plants were removed from the C.E. and the regrowth over the seven days measured and recorded to the nearest mm. Regrowth was measured from the point of cut to the tip of regrown leaf using a ruler. The plants were returned to the C.E. and 14 days after the re-trimming of the shoots, the length of the regrowth was again measured. The regrowth was then cut with scissors at the point of the original cuts and the new growth

of each shoot was weighed accurately to three decimal places. Any plants which had become submerged and died were discounted and excluded from the results.

Results

Regrowth of leaves of the susceptible Rothamsted was much less than for the resistant Woburn population (Tables 1 and 2, Figure 1). There was no evidence that measuring regrowth after 14 days provided a better discrimination between populations than after 7 days. The weight of regrowth (difficult to measure due to the very small amounts of foliage) showed no advantage over shoot length assessments (Table 3).

Table 1. Shoot regrowth (mm) after 7 days

	Number of plants		Mean regrowth (mm)		% of Nils	
fenoxaprop	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	10	12	22.8	20.8	100	100
0.024 ppm	5	7	22.0	21.3	96	102
0.074 ppm	7	7	11.6	21.3	51	102
0.222 ppm	6	7	6.3	24.6	28	118
0.666 ppm	8	7	6.0	16.7	26	80
2 ppm	6	6	3.3	12.7	15	61

Table 2. Shoot regrowth (mm) after 14 days

	Number of plants		Mean regrowth (mm)		% of Nils	
fenoxaprop	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	10	12	30.0	33.3	100	100
0.024 ppm	5	7	25.0	32.6	83	98
0.074 ppm	7	7	12.9	29.0	43	87
0.222 ppm	6	7	8.3	35.0	28	105
0.666 ppm	8	7	7.5	22.1	25	66
2 ppm	6	6	4.7	21.7	16	65

Table 3. Weight (g) of shoot regrowth after 14 days

	Number of plants		Mean regrowth (g)		% of Nils	
fenoxaprop	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	10	12	0.009	0.017	100	100
0.024 ppm	5	7	0.100	0.008	111	46
0.074 ppm	7	7	0.004	0.010	48	59
0.222 ppm	6	7	0.002	0.012	20	73
0.666 ppm	8	7	0.001	0.009	11	56
2 ppm	6	6	0.003	0.010	33	57

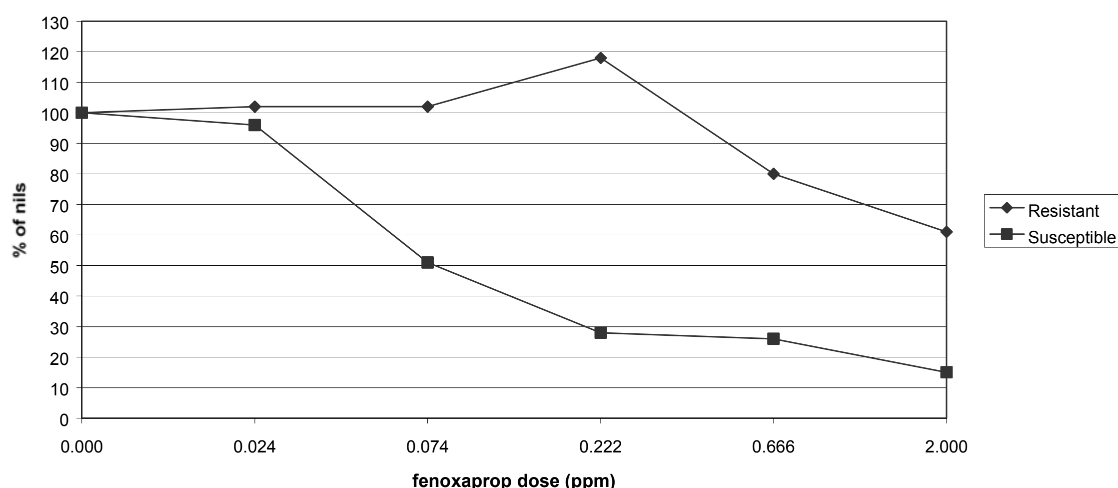


Figure 1. Length of shoot regrowth after 7 days (excluding submerged plants)

Shoot regrowth data after 7 days (Table 1; Figure 1) were analysed using a Maximum Likelihood Programme and $\log_{10}$ ED_{50} values calculated. ED_{50} values were detransformed from the $\log_{10}$ data and represent the herbicide dose required to decrease shoot length by 50% relative to the no-herbicide controls. The $\log_{10}$ ED_{50} values were Rothamsted -1.0126 and Woburn 0.3743 (SED ± 0.2642). Back transformed ED_{50} values (ppm fenoxaprop) were Rothamsted 0.0972 and Woburn 2.3673, giving a resistance index (ratio of ED_{50} values) of 24.4. Consequently, differences in response to fenoxaprop between the two populations were clearly demonstrated.

Conclusions

- **The technique is capable of detecting resistance to fenoxaprop in plants from the field. The most discriminating single dose appeared to be either 0.222 or 0.666 ppm.**

- **Plants from the field were much more variable in size than typical pot grown plants. Consequently, trimming shoots to a prescribed length is inappropriate and trimming to just below the axil of the longest leaf results in better standardisation.**
- **10 ml of solution is too much for small plants, resulting in some becoming submerged. 5 ml may be better - see next experiment.**
- **There is no advantage in recording shoot recovery after 14 as compared with 7 days with fenoxaprop.**
- **Measuring shoot weight shows no advantage over shoot length measurements.**

EXPERIMENT 2: DOSE RESPONSE ASSAY WITH SETHOXYDIM.

Materials and Methods

The technique used was similar to that in Experiment 1. Black-grass plants (Rothamsted (= susceptible, S) and Woburn (= resistant, R) populations) at the 3 leaf to 5 tiller stage were dug up on 17 January 2001, roots were then washed to remove soil before being trimmed to 50 mm.

The experiment with sethoxydim was started on 18 January 2001 using the formulated product 'Checkmate' (193 g a.i. per L) with eight replicate plants per population at each of four doses, (0.024, 0.222, 2.0, 18 ppm), and 12 untreated control plants per population in demineralised water. These doses were formed in a dilution series as in Experiment 1 using demineralised water. Again a double dilution technique was used with 0.65ml of the commercial herbicide mixed in 500 ml of water, and then 36 ml of this solution made up to 500 ml with demineralised water to produce an 18 ppm sethoxydim solution. To produce lower concentrations, 55.6 ml of the 18 ppm solution was made up to 500 ml with demineralised water to produce a 2 ppm solution, and this step repeated to produce the 0.222 and 0.024 ppm solutions.

Solutions were decanted into small plastic vials each holding 5 ml solution (instead of the 10 ml used in Experiment 1), with 8 vials per dose per population and 12 control vials per population. Plants were placed in the appropriate vials and were *not* trimmed initially, in contrast to Experiment 1. The plants were transferred to a controlled environment (C.E.) cabinet set to provide a 14-hour (16°C) day and a 10-hour (10°C) night. After 24 hours in the C.E. cabinet the plants were removed, trimmed across all their leaves with scissors at a point 1-2mm below the axil of their upper leaf, then returned to the C.E.

Seven and 14 days after trimming, the plants were removed from the C.E. and the regrowth measured and recorded to the nearest mm as in Experiment 1. After 14 days, the regrowth was cut with scissors at the point of the original cut and the new growth of each shoot was weighed accurately to three decimal places. Any plants which had become submerged and died were discounted and excluded from the results.

Results

As with fenoxaprop, regrowth of leaves of the susceptible Rothamsted was much less than for the resistant Woburn population (Tables 4 and 5), although the effects were quantitative, not qualitative. Again, there was no evidence that measuring regrowth after 14 days provided a better discrimination between populations than after 7 days. The weight of regrowth (Table 6) was difficult due to the very small amounts of foliage and showed no advantage over shoot length assessments.

Table 4. Shoot regrowth (mm) after 7 days

	Number of plants		Mean regrowth (mm)		% of Nils	
Sethoxydim	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)
Nil	12	9	20.2	25.2	100	100
0.024 ppm	8	6	23.6	23.3	117	93
0.222 ppm	8	7	10.4	17.4	51	69
2 ppm	8	8	5.3	18.0	26	71
18 ppm	8	8	3.6	9.8	18	39

Table 5. Shoot regrowth (mm) after 14 days

	Number of plants		Mean regrowth (mm)		% of Nils	
Sethoxydim	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)
Nil	12	9	23.6	30.1	100	100
0.024 ppm	8	6	25.3	21.8	107	73
0.222 ppm	8	7	12.3	19.9	52	66
2 ppm	8	8	5.5	22.1	23	73
18 ppm	8	8	3.6	12.6	15	42

Table 6. Weight (g) of shoot regrowth after 14 days

	Number of plants		Mean regrowth (g)		% of Nils	
Sethoxydim	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)	Roth. (S.)	Woburn (R)
Nil	12	9	0.008	0.010	100	100
0.024 ppm	8	6	0.011	0.008	138	80
0.222 ppm	8	7	0.001	0.005	13	50
2 ppm	8	8	0.001	0.006	13	60
18 ppm	8	8	0	0.004	0	40

Shoot regrowth after 7 days data (Table 4) were analysed using a Maximum Likelihood Programme and $\log_{10}$ ED_{50} values calculated. ED_{50} values were detransformed from the $\log_{10}$ data and represent the herbicide dose required to decrease shoot length by 50% relative to the no-herbicide controls. The $\log_{10}$ ED_{50} values were: Rothamsted -0.3710, Woburn 0.6099, SED ± 0.6592. Detransformed ED_{50} values (ppm sethoxydim) were: Rothamsted 0.4256, Woburn 4.0731, giving a resistance index: (ratio of ED_{50} values) of 9.6. Consequently differences in response to sethoxydim between the two populations were demonstrated, but the differences were not as large as for fenoxaprop.

It was evident that the amount of regrowth varied considerably between individual Woburn plants. This is not unexpected as only some of the plants of the Woburn population have target site resistance and consequently would show resistance to sethoxydim. Figure 2 presents the results in terms of proportion of plants with a shoot regrowth length of over 10 mm after 7 days. There were large differences between the two populations, especially at the two highest doses.

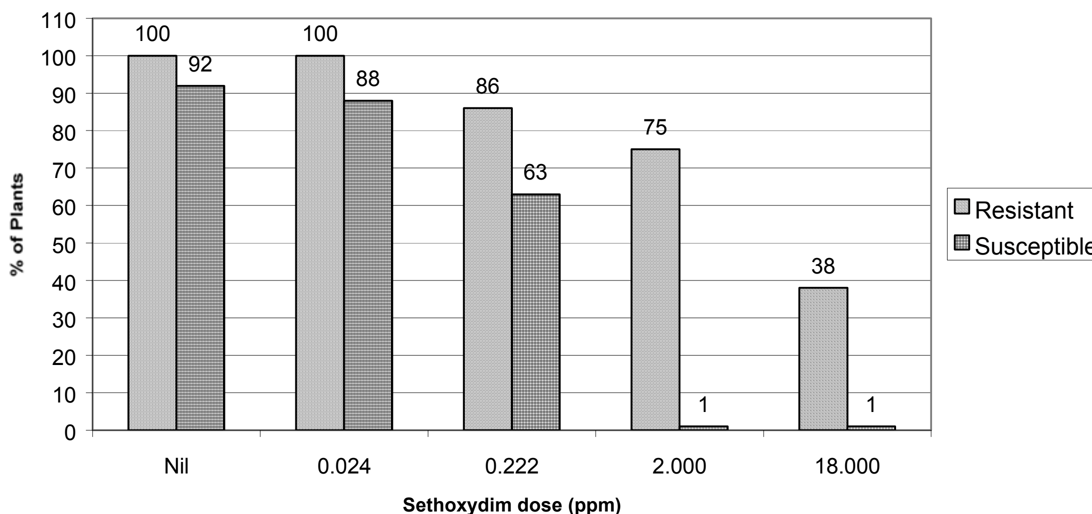


Figure 2. Percentage of plants with regrowth of ≥ 10 mm after 7 days exposure to sethoxydim.

Conclusions

- **The technique is capable of detecting resistance to sethoxydim in plants from the field. The best discriminating single dose is 2 ppm.**
- **Trimming plants once, 24 hours after they have been placed in herbicide solution, appears to be a satisfactory alternative to trimming immediately after placing plants in herbicide solution, and again after 24 hours (as done in experiment 1). This saves time.**
- **5 ml of herbicide solution appears adequate and reduces the risk that small plants become submerged and killed.**
- **There is no advantage in recording shoot recovery after 14 days rather than 7 days after treatment with sethoxydim.**
- **Measuring shoot weight shows no clear advantage over shoot length measurements with sethoxydim.**

EXPERIMENT 3: IDENTIFICATION OF BEST SINGLE DISCRIMINATING DOSE FOR FENOXAPROP AND SETHOXYDIM.

Materials and Methods

The aim was to find a single herbicide dose of fenoxaprop and sethoxydim that would best discriminate between resistant and susceptible black-grass. The three black-grass populations used were **Rothamsted** 1999 (= susceptible, S), **Peldon** 1996 (resistant by enhanced metabolism) and **Notts**. A1 1993 (resistant by an insensitive ACCase target site). The original intention had been to collect plants from fields on the relevant farms, but foot and mouth restrictions meant that this was not possible. However, the plants were grown in an agricultural soil in the outdoor containers which closely mimic true field conditions. Plants at the 2 tiller stage were dug up on 31 January 2001 from outdoor containers sown in the previous autumn. Roots of these seedlings washed to remove soil and trimmed to 50 mm.

The following concentrations were used: fenoxaprop-P-ethyl at 0.222 and 0.666 ppm; sethoxydim at 2 and 6 ppm. The experiment was started on 1 February with 12 replicate plants for Rothamsted and 10 replicate plants for the other two populations at each dose, and 12 untreated control plants per population in demineralised water. 2.28ml of the commercial fenoxaprop product ("Cheetah Super" 55 g a.i./L) was measured out with a disposable syringe and mixed in 500 ml of water in a measuring cylinder. 1.34 ml of this solution was then measured out with another syringe and made up to 500 ml with demineralised water in a second measuring cylinder to produce a 0.666 ppm solution. To produce the 0.222 ppm concentration, 167 ml of the 0.666 ppm solution was made up to 500 ml with demineralised water. For sethoxydim, 0.65 ml of the commercial product ("Checkmate" 193 g a.i./L) was mixed in 500 ml water, and then 12 ml of this solution mixed into 500 ml of demineralised water in a second cylinder to produce the 6 ppm solution. To produce the 2 ppm concentration, 167 ml of the 6 ppm solution was made up to 500 ml with demineralised water.

As in Experiment 2, solutions were decanted into small plastic vials each holding 5 ml solution (instead of the 10 ml used in Experiment 1), with 10 or 12 vials per dose per population and 12 control vials per population containing demineralised water. Plants were placed in the appropriate vials and were *not* trimmed initially. The plants were transferred to a controlled environment (C.E.) cabinet set to provide a 14-hour (16°C) day and a 10-hour (10°C) night. After 24 hours in the C.E. cabinet the plants were removed, trimmed across all

their leaves with scissors at a point 1-2mm below the axil of their upper leaf (as in Experiment 2), then returned to the C.E.

Seven and 11 days after trimming, the plants were removed from the C.E. and the regrowth measured and recorded to the nearest mm as in Experiment 1. After 11 days, the regrowth was cut with scissors at the point of the original cut and the new growth of each shoot was weighed accurately to three decimal places. Any plants which had become submerged and died were discounted and excluded from the results.

Results

Shoot regrowth after 7 days with both fenoxaprop treatments was greatest with Notts., least with Rothamsted with Peldon intermediate (Table 7). Shoot length relative responses after 11 days were very similar to 7 days and are not presented. This was consistent with the fenoxaprop resistance ratings for these populations found in previous whole plant screening assays: Notts. RRR; Peldon RR; Rothamsted S (susceptible) (Moss *et al*., 1999). Differences were also clearly detected between the shoot weights after 11 days (Table 8), but fenoxaprop at 0.666 ppm appeared to be a better discriminating dose than 0.222 ppm. At this dose, the mean shoot lengths (with S.E. ±) were: Rothamsted 9.2 mm ± 1.093; Peldon 17.7 ±2.014; Notts. 36.4 ± 7.070. The differences between each pair of populations were statistically significant ($P \leq 0.05$).

Table 7. Shoot regrowth (mm) after 7 days

	Number of plants			Mean regrowth (mm)			% of Nils		
	Roth.	Peld.	Notts.	Roth.	Peld.	Notts.	Roth.	Peld.	Notts.
Nil	11	12	12	23.5	34.8	32.8	100	100	100
fenoxaprop									
0.222 ppm	11	10	10	13.6	31.7	42.2	58	91	129
0.666 ppm	12	9	10	9.2	17.7	36.4	39	51	111
sethoxydim									
2 ppm	12	10	9	6.3	5.3	36.7	27	15	112
6 ppm	12	10	10	7.8	3.8	32.3	33	11	98

Table 8. Weight (g) of shoot regrowth after 11 days

	Number of plants			Mean weight (g)			% of Nils		
	Roth.	Peld.	Notts.	Roth.	Peld.	Notts.	Roth.	Peld.	Notts.
Nil	11	12	12	0.018	0.028	0.022	100	100	100
Fenoxaprop									
0.222 ppm	11	10	10	0.007	0.028	0.024	39	100	109
0.666 ppm	12	9	10	0.003	0.012	0.029	17	43	132
Sethoxydim									
2 ppm	12	10	9	0.004	0.003	0.023	22	11	105
6 ppm	12	10	10	0.005	0.002	0.019	28	7	86

Sethoxydim had very little effect on either shoot regrowth or weights for Notts., which is entirely consistent with the high level of target site resistance (insensitive ACCase) in this population. The other two populations, Rothamsted and Peldon, do not possess target site resistance and would both be expected to be susceptible to sethoxydim. The Peldon population was at least as susceptible to sethoxydim as the Rothamsted susceptible standard both in terms of shoot regrowth and weights. Sethoxydim at 2 ppm appeared to be suitable as a single discriminating dose. At this dose, the mean shoot lengths (with S.E. ±) were: Rothamsted 6.3 mm ± 1.130; Peldon 5.3 ± 0.857; Notts. 36.7 ± 5.472. The differences between Notts. and the other two populations were statistically significant ($P \leq 0.05$).

Using the "R" rating system for allocating different degrees of resistance to populations (Moss *et al.*, 1999) gave the following results using shoot regrowth after 7 days: fenoxaprop 0.666 ppm - Peldon RR, Notts. RRR; sethoxydim 2 ppm - Peldon S, Notts RRR. These ratings are consistent with previous whole plant screening studies.

Conclusions

- **The technique is capable of detecting resistance to fenoxaprop and sethoxydim in black-grass plants from the field.**
- **The best discriminating single dose of fenoxaprop is 0.666 ppm.**
- **The best discriminating single dose of sethoxydim is 2 ppm.**
- **Measuring shoot weight shows no clear advantage over shoot length measurements with either fenoxaprop or sethoxydim.**

EXPERIMENTS 4 & 5: CONFIRMATION OF BEST SINGLE DISCRIMINATING DOSE FOR FENOXAPROP AND SETHOXYDIM.

Materials and Methods

The aim was to confirm that the single herbicide doses of fenoxaprop and sethoxydim identified in the previous experiments were the best for discriminating between resistant and susceptible black-grass. The two black-grass populations used were **Rothamsted** (= susceptible, S) and **Woburn (R)**, as used in experiments 1 & 2. Plants in the field at the 2 - 5 tiller stage were dug up on 27 February 2001 (Experiment 4) and 3 April 2001 (Experiment 5). Roots of these seedlings washed to remove soil and trimmed to 50 mm.

The following concentrations were used: fenoxaprop-P-ethyl at 0.222 and 0.666 ppm; sethoxydim at 2 and 6 ppm. Both concentrations were used in Experiment 4, but only 0.666 ppm fenoxaprop and 2 ppm sethoxydim in Experiment 5. There were 12 (Experiment 4) or 18 (Experiment 5) replicate plants per herbicide dose for both populations and 15 untreated control plants per population in demineralised water.

The herbicide mixing procedures were the same as in Experiment 3, although in Experiment 5 the second dilution for sethoxydim involved 4 ml, rather than 12 ml so that 2 ppm solution could be produced directly. As in experiments 2 & 3, solutions were decanted into small plastic vials each holding 5 ml solution (instead of the 10 ml used in Experiment 1). Plants were placed in the appropriate vials and were *not* trimmed initially. The plants were transferred to a controlled environment (C.E.) cabinet set to provide a 14-hour (16°C) day and a 10-hour (10°C) night. After 24 hours in the C.E. cabinet the plants were removed, trimmed across all their leaves with scissors at a point 1-2mm below the axil of their upper leaf (as in Experiments 2 & 3), then returned to the C.E.

Seven days (and 14 days in Experiment 4) after trimming, the plants were removed from the C.E. and the regrowth measured and recorded to the nearest mm as in previous experiments. Any plants which had become submerged and died were discounted and excluded from the results.

Results

In Experiment 4, shoot regrowth after both 7 and 14 days with the fenoxaprop and sethoxydim treatments was greater with Woburn than with the susceptible Rothamsted

(Tables 9 & 10). This was consistent with previous experimental results. The relative differences were greater after 14 days than 7 days with fenoxaprop, but not with sethoxydim. It appeared that the fenoxaprop treated Woburn shoots continued to grow after 7 days, whereas the Rothamsted shoots did not. With both fenoxaprop and sethoxydim, there was no advantage of either the higher or lower dose in terms of discriminating between resistant and susceptible plants.

An alternative way of interpreting the responses of the plants is in terms of the number of shoots which have regrown by at least 10 mm after 7 days. On this basis, with fenoxaprop at 0.666 ppm, two out of 12 Rothamsted (17%) and nine out of 12 Woburn (75%) had shoot lengths of 10 mm or more after 7 days. With sethoxydim at 2 ppm, no Rothamsted (0%) but five out of 12 Woburn (42%) had shoot lengths of 10 mm or more after seven days.

Table 9. Experiment 4: Shoot regrowth (mm) after 7 days

	Number of plants		Mean regrowth (mm)		% of Nils	
	Roth. (S)	Woburn (R).	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	15	15	27.9	32.8	100	100
Fenoxaprop						
0.222 ppm	12	12	8.3	18.6	30	57
0.666 ppm	12	12	6.6	16.7	24	51
Sethoxydim						
2 ppm	12	12	4.0	14.8	14	45
6 ppm	12	12	4.8	14.8	17	45

Table 10. Experiment 4: Shoot regrowth (mm) after 14 days

	Number of plants		Mean regrowth (mm)		% of Nils	
	Roth. (S)	Woburn (R).	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	15	15	38.7	36.1	100	100
Fenoxaprop						
0.222 ppm	12	12	8.8	23.6	23	65
0.666 ppm	12	12	6.8	20.9	17	58
Sethoxydim						
2 ppm	12	12	4.4	16.8	11	46
6 ppm	12	12	4.9	16.9	13	47

In Experiment 5, shoot regrowth values after 7 days (Table 11) were very similar to those in Experiment 4, demonstrating the consistency of the technique. The "R" rating system (Moss *et al.*, 1999), gave the following results: fenoxaprop 0.666 ppm - Woburn RR; sethoxydim 2 ppm - Woburn RR. These ratings are consistent with previous whole plant screening studies.

The number of shoots which had regrown by at least 10 mm after 7 days was: fenoxaprop at 0.666 ppm, four out of 18 Rothamsted (22%) and 13 out of 18 Woburn (72%); sethoxydim at 2 ppm, no Rothamsted (0%) but seven out of 18 Woburn (39%). A previous estimate of the frequency of target site-resistant plants within the Woburn population was 36%, so the proportion of plants with shoot regrowth of 10 mm or more in Experiments 4 (42%) & 5 (39%) are in good agreement with this estimate, especially bearing in mind the small sample sizes.

Table 11. Experiment 5: Shoot regrowth (mm) after 7 days

	Number of plants		Mean regrowth (mm)		% of Nils	
	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)	Roth. (S)	Woburn (R)
Nil	15	15	36.1	34.7	100	100
Fenoxaprop						
0.666 ppm	18	18	7.6	18.3	22	51
Sethoxydim						
2 ppm	18	18	3.1	14.3	9	40

Conclusions

- The technique is consistent in detecting resistance to fenoxaprop and sethoxydim in black-grass plants from the field.
- The best discriminating single dose of fenoxaprop is 0.666 ppm.
- The best discriminating single dose of sethoxydim is 2 ppm.
- Measuring shoot length after 14 days can, in some instances, give better results than after 7 with fenoxaprop, but not with sethoxydim.
- Assessing the proportion of shoots which had regrown by 10 mm or more after 7 days gave additional information and enabled better discrimination between resistant and susceptible populations.
- The "R" rating system previously used in pot and petri-dish assays (Moss, *et al*, 1999) also appears appropriate for use with shoot regrowth data obtained after 7 days.

PROTOCOL FOR TESTING BLACK-GRASS PLANTS COLLECTED FROM THE FIELD.

It is absolutely essential that plants of a known susceptible standard are available, ideally at a similar growth stage to the populations under test. Preferably, standard resistant populations will also be available, but these are not essential. Standard populations may be grown in field plots or in large outdoor containers.

Sample collection

1. **Dig up plants in the field with a trowel, leaving a small amount of soil around the roots. Minimise damage to the roots and shoots. Aim to collect 50 - 100 plants per sample, collected from a representative area within the field. Prevent the samples drying out, but do not keep them in excessively wet conditions.**

Sample preparation

2. **Prepare samples as soon as possible after collection. If they need storing, keep them in a cool, lit place and prevent them drying out. Plants can be stored in trays outdoors for at least 14 days. Wash as much soil as possible from the roots and then trim the root system to 5 cm using scissors or a scalpel. If plants have tillered, split into single tiller samples, discarding any which possess few roots. Place plants in a shallow tray of water to prevent drying out. Repeat with all populations, including the susceptible standard. Ensure samples are labelled and kept separate.**

Preparing vials

3. **Label vials (10 ml size ideal) with population name and herbicide dose. A minimum of 10 and preferably 20 individual plants per population should be used at each herbicide dose, and for untreated controls. Arrange vials in a logical order in a suitable container that will prevent them being knocked over (polystyrene modular seedling trays are ideal). Add 5 ml de-mineralised water to each control vial.**

Preparing herbicide solutions (fenoxaprop and sethoxydim)

4. **Wearing appropriate safety protection (principally faceshield, gloves and protective clothing) measure out 2.28ml of a commercial formulation of a product containing 55 g a.i./litre of fenoxaprop-P-ethyl (e.g. "Cheetah Super") using a 5 ml disposable syringe and mix into 500 ml of demineralised water in a measuring cylinder. After thorough mixing, measure out 1.34 ml of this solution using a 2 ml syringe, and mix**

into 500 ml of demineralised water in a *second* measuring cylinder. This produces a 0.666 ppm fenoxaprop solution. Measure out 5 ml of this solution into each fenoxaprop vial using either a 5 ml syringe or a dispenser.

With sethoxydim, measure out 0.65 ml of a commercial formulation containing 193 g sethoxydim/litre (e.g. "Checkmate") using a 1 ml disposable syringe and mix into 500 ml of demineralised water in a measuring cylinder. Then measure out 4 ml of this solution using a 5 ml syringe and mix into 500 ml of demineralised water in a *second* cylinder. This produces a 2 ppm sethoxydim solution. Measure out 5 ml of this solution into each sethoxydim vial using either a 5 ml syringe or a dispenser.

5. Placement of plant samples into vials

Take the plant samples and remove excess water from the roots using tissue paper. Place one plant in each labelled vial, ensuring that roots are submerged in the solutions. Randomise and place tray of plants in a controlled environment cabinet (or a glasshouse) set to provide a 14-hour (16°C) day and a 10-hour (10°C) night. After 24 hours in the C.E. cabinet, trim plants across all their leaves with scissors at a *point 1-2mm below the axil of the upper leaf.*

In some cases, such as with large plants, it may be helpful to trim them immediately before they are placed in the vials. If this is done, then plants should be trimmed again to the point of the original cut, 24 hours after the original trimming.

6. Plant assessments

Seven days after trimming (or retrimming), measure leaf regrowth from the point of the original cut, which will still be easily visible. Use a ruler and record to nearest mm. Ignore any growth from newly formed tillers. Discount any plants which have died due being submerged or through not having their roots in the solutions.

Recording leaf regrowth after 7 days is likely to be the single most appropriate assessment. However, in some cases it may be advantageous to record after 14 days in order to improve discrimination between populations. The proportion of leaves with regrowth of more than 10 mm is also a good means of improving discrimination between resistant and susceptible populations. Weight of regrowth may be useful in some cases, although this requires the use of a 3 decimal place balance due to the very small amounts of foliage being weighed.

7. Interpretation of results

Calculate mean regrowth for each treatment for each population and compare with the results for the susceptible standard. Expressing the results in terms of % of untreated (Nil) values for shoot length is useful as a means of removing the effects of differential growth rates *not* associated with herbicide treatments. Regrowth in untreated controls will give an indication of the scale of any such effects. If there are large differences in potential regrowth, as seen between untreated controls, treat any treatment differences between populations with caution.

The "R" rating system previously used in pot and petri-dish assays also appears to be appropriate for use with shoot regrowth data obtained after 7 days. Note that this uses % *reduction* values in shoot growth regrowth relative to untreated controls. For any one treatment, this is:

$$\frac{\text{Mean regrowth in untreated} - \text{Mean regrowth in treated}}{\text{Mean regrowth in untreated}} \times 100$$

This system was described in detail by Moss, *et al*, (1999) and is reproduced here with minor modifications:

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 reasonably high, preferably over 80%. The % reduction values between the susceptible standard and zero are separated into five equal categories (see Figure 3). 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).

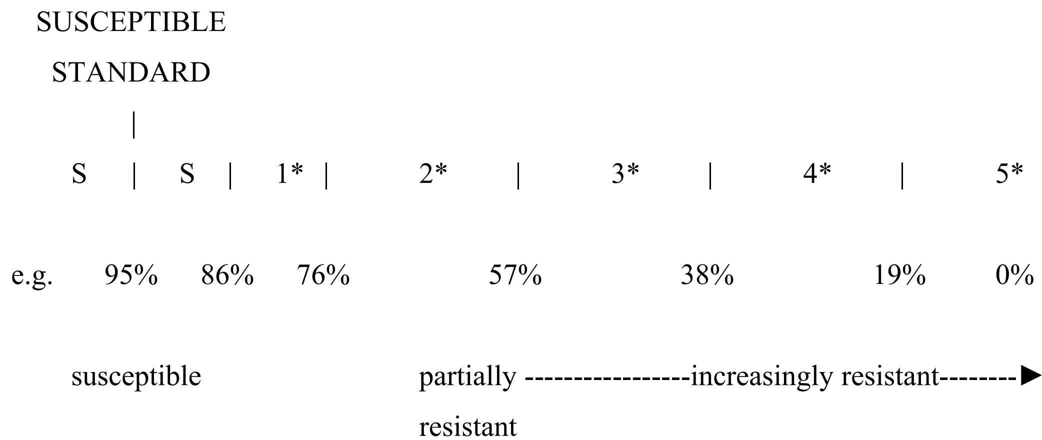


Figure 3. Categories of resistance ratings.

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.

7. OVERALL CONCLUSIONS

The validation exercise undertaken at IACR - Rothamsted concentrated on the most economically important weed species (black-grass) and the most widely used herbicide mode of action (ACCase inhibitors). The validation exercise identified the optimum discriminating doses of fenoxaprop-p-ethyl and sethoxydim for use in herbicide resistance detection assays and includes a protocol for the implementation of these assays (Section 6). Because of the foot and mouth epidemic these protocols were only tested on one wild resistant black-grass population (Woburn) but the doses were identified using two resistant populations (Peldon and Notts) which were grown under field conditions at IACR - Rothamsted.

These verification studies show that the herbicide resistance detection assays developed at IACR - Long Ashton Research Station were effective in the case of black-grass. The assays developed for Italian rye-grass and wild-oats were not verified due to time/financial constraints but present an avenue for further study in the light of the successful validation of the black-grass herbicide resistance test. The protocols described in Section 6 enable black-grass plants collected in the crop to be reliably tested for resistance to ACCase inhibiting herbicides. The benefits of the protocol described here compared to spraying pot grown plants and assessing phytotoxicity include: -

- no need to allow suspected herbicide resistant plants to produce seed
- test may be performed easily in the growing season
- time taken for test (one to two weeks) is shorter
- area of glasshouse or controlled environment room occupied by test is far less (100 tubes $0.1m^2$ 100 pots $1.0m^2$)

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APPENDIX A

Title of Bursary project:

Influence of herbicide chemistry on the effectiveness of bioassays to detect ACCase resistance resulting from enhanced metabolism.

Ongoing HGCA-funded project: **No.:** 2144

The development of a rapid bioassay for the detection of herbicide resistance in wild-oat, black-grass and Italian rye-grass at various growth stages.

Student: Rachael Pell. **Supervisor:** Andrew Wilson

ABSTRACT

Herbicide resistant Black-grass was first detected in the UK in 1982 and has since been found in Italian rye-grass and Wild-oat. The ongoing HGCA project is developing a rapid bioassay for the detection of herbicide resistance. ACCase inhibiting herbicides differ in their phytotoxicity to individual grasses. The object of this study was to enhance the existing test for discriminating between susceptible (S) and metabolism based resistant (R) biotypes for ACCase inhibiting herbicides by identifying the most effective herbicide for each species. The rapid bioassay method was used to distinguish between R and S biotypes of Black-grass, Wild-oat and Italian rye-grass at seedling and tillering growth stages. Trimmed seedlings and tillers were immersed in dose ranges of fenoxaprop-P-ethyl, clodinafop-propagyl, diclofop-methyl and tralkoxydim that had been determined in the initial stages of the bursary project. The length and fresh weight of new shoot growth of trimmed propagules were used to discriminate between R and S biotypes. Discrimination between biotypes was verified using non-linear regression analysis and test for lack-of-fit. I_{50} values (herbicide dose required to inhibit growth by 50%) for shoot length were determined and were closely correlated to those for fresh weight. Fenoxaprop-P-ethyl was identified as the most effective herbicide, overall, for detection of ACCase inhibitor resistance in the three weed species, although diclofop-methyl and tralkoxydim provided clear resolution between S and R biotypes of Wild-oat and Italian rye-grass.

APPENDIX B

IACR / HGCA RESISTANCE DRAFT TEST PROTOCOL (MULTIPLE DOSE)

1. SAMPLE COLLECTION

Once a suspected resistant weed population of black-grass, Italian rye-grass or wild-oat has escaped a herbicide treatment, samples need collecting as quickly as possible. Using a trowel, dig up the weed leaving a small block of soil around the base of the weed. Great care needs to be taken not to damage the aerial parts of the weed. 30 to 60 propagules are needed for the formation of dose-response curves.

2. SAMPLE PREPARATION

a) Seedling Test

Handling samples carefully, as much of the soil as possible needs to be removed from the root system. This is best done by placing under a slow running tap and gently rubbing the root system. Then using a razor blade, trim the root system to 5 cm. Then place the root system in a shallow tray of water and gently 'brush' as much as the remaining soil from the root system as is possible using fingers. Repeat this for all of the samples and the known susceptible samples[1]. Rest the samples on the edge of the tray with the root systems still immersed. Ensure sample groups are not mixed up so keep in different trays and always keep a label with them. The seedling test can be conducted on all weed species until the 2–3 tiller growth stage. Carefully remove all tillers (if there are any) at this stage.

b) Tiller Test.

Treat plant as described for the seedling test. After trimming the root system to 5 cm, the tillers need separating from the main stem. This can be done by carefully teasing them from the main stem. It is suggest that tillers of the 3-4 leaf stage are used as propagules need to be as uniform as possible. Once removed the remaining soil can then be washed off the tiller root system

3. PREPARING VIALS

Label the 25 ml glass vials with the population name and the herbicide dose (this can be substituted with a number for ease) together with the replication number. Labelling should be done clearly on the side of the vial. A minimum of six replications per dose should be employed. Place on a tray in rows of similar treatments starting with the controls. Leave a space between treatments so that no cross contamination occurs when filling the vials with herbicide solution. Then using a 50 ml syringe add 15 ml deionised water to each control vial.

[1] Confirmed susceptible/resistant biotypes of the same species need to be available at a similar growth stage so that comparisons maybe made.

4. PREPARING AND ADDING HERBICIDE SOLUTION

Example: Isoproturon seedling test (2-3 leaf) *Lolium multiflorum*.

Half fill a 250 ml measuring cylinder with deionised water, then measure 0.25 ml of a product containing 500g/litre isoproturon using a 1 ml syringe, add this to the water and make up to 250 ml. Mix thoroughly by gentle inversion and label the cylinder. This results in a stock solution of 500 mg a.i./litre from which subsequent dilutions are to be made.

These calculations are from the attached example using 8 replications per dose. Using a pipette take 0.094 ml of stock solution and place in a 500 ml conical flask and make up to 250 ml with deionised water, mix thoroughly and decant into a suitable beaker, this solution contains 0.188 mg a.i./litre isoproturon, add 15 ml solution to each vial. If there is any excess solution dispose of in a safe manner. Next add 0.188 ml of stock and make up to 250 ml, this contains 0.375 mg a.i./litre dispense into vials as before. Continue in this manner multiplying the amount of stock added by two and making up to 250 ml, always moving up the concentration gradient, until all of the vials have been filled.

Placement of weed samples into vials

Take the weed samples and trim the shoots to 5 cm in length. Keeping groups of samples separate, gently place on tissue paper and remove any excess water, do not press too hard. Then place one plant in every vial making sure the correct samples are placed in the correct vials, error at this stage will result in misleading results. Randomise and place tray containing vials in glasshouse or in controlled environment (17°C 16 hour day, 10°C 8 hour night). After 24 hours carefully re-trim the test material to the original cut off point.

5. SAMPLE ASSESSMENT

Assays with ACCase inhibiting herbicides can be assessed after 7 days. For assays with PS2 inhibiting herbicides, seedling tests can be assessed after 7 days, however, it is recommended that tiller tests are left for 10 days before assessment. De-randomise (carefully so as not to damage plant material) and drain the herbicide solution into a suitable receptacle, partially refill with water, gently agitate with the plant still in the vial and drain again. If the new growth is necrotic, likely in higher concentrations of PS2 inhibitor assays, do not get it wet as this will lead to a weight increase. Then place a small amount of water in the vials.

It may be easier to remove the plant material from the vial before assessment. When assessing, use a razor to cut at the point of the original cut (which will be clearly visible). Use a ruler to measure length of new shoot growth from the cut off point record this then weigh the new shoot growth on a balance with 3 decimal places and record it. Ignore any growth which as arisen from a newly formed tillers.

6. DATA ANALYSIS

Results are subject to ANOVA following a suitable transformation (usually $\log_e$) then non-linear regression analysis using lack-of-fit test is employed to determine dose-response curves and LD_{50}'s.

Suggested dose ranges for assays for the detection of herbicide resistance.

Species	Test	Herbicide	Dose range (mg a.i./litre)
BG IRG	ST	Isoproturon	0, 0.188, 0.375, 0.75, 1.5 and 3.
BG IRG	TT	Isoproturon	0, 0.75, 1.5, 3, 6, and 12.
BG	ST	Chlorotoluron	0, 0.16, 0.8, 4, 20 and 100.
BG	TT	Chlorotoluron	0, 0.16, 0.8, 4, 20 and 100.
All	ST	fenoxaprop-P-ethyl	0, 0.024, 0.074, 0.222, 0.666 and 2.
BG IRG [1]	TT	fenoxaprop-P-ethyl	0, 0.024, 0.074, 0.222, 0.666 and 2.
All	ST	Diclofop-methyl	0, 0.1, 0.3, 0.88, 2.67 and 8.
IRG	TT	Diclofop-methyl	0, 0.1, 0.3, 0.88, 2.67 and 8.
IRG WO	ST	Tralkoxydim	0, 0.062, 0.185, 0.556, 1.667 and 5.
IRG	TT	Tralkoxydim	0, 0.062, 0.185, 0.556, 1.667 and 5.

Example.

Species: Italian rye-grass (*Lolium multiflorum*).

Herbicide: Isoproturon.

Test: Seedling test (2-3 leaf).

Susceptible (Halja) and Resistant (Clev) Biotypes of *L. multiflorum* sown 03/07/00, pricked out 10/07/00 in 9 cm diameter pots containing sandy loam soil amended to grit and transferred to glasshouse. Plants 'harvested' at 2-3 leaf growth stage. Soil carefully removed from root system and plants trimmed to 5 cm shoot and root length.

Dose range employed: 0, 0.188, 0.375, 0.75, 1.5 and 3 mg a.i. L^{-1}.
15 ml herbicide solution placed in 25 ml glass vials with 8 replications per dose. Thus need 15 x 8 x 2 ml herbicide solution per dose.
Stock solution: 0.25 ml isoproturon (formulated as 'stress' – 500g/l) made up to 250 ml = 500 mg a.i./litre.

1.5 ml stock made up to 250 ml = 3 mg a.i. L^{-1}.
0.75 ml stock made up to 250 ml = 1.5 mg a.i. L^{-1}.
0.375ml stock made up to 250 ml = 0.75 mg a.i. L^{-1}.
0.188 ml stock made up to 250 ml = 0.375 mg a.i. L^{-1}.
0.094 ml stock made up to 250 ml = 0.188 mg a.i. L^{-1}.

Vials labelled 1-12 (i – viii).
Seedlings placed in vials, experiment randomised and placed in CE cabinet set at 17°C 16 hour day 10°C 8 hour day. Seedling re-trimmed to original point of trimming after 24 hours. Experiments assessed 7 days after initial treatment. Lengths and fresh weights of new shoot growth recorded (see below).

Results.

Susceptible					Resistant			
ipu	rep	total new	Fwt new		ipu	rep	shoot	fwt new
conc (mg/l)		Growth	Growth (g)		conc (mg/l)		length (cm)	growth (g)
0	1	6.1	0.018		0	1	4.9	0.021
0	2	2.6	0.007		0	2	5.2	0.017
0	3	4.5	0.01		0	3	5.2	0.016
0	4	6	0.019		0	4	7.5	0.029
0	5	5.9	0.017		0	5	2.9	0.009
0	6	5.2	0.024		0	6	6.4	0.015
0	7	6.3	0.029		0	7	7.8	0.03
0	8	6.5	0.022		0	8	5.8	0.017
ave		**5.4**	**0.018**		**ave**		**5.7**	**0.019**
0.188	1	0.6	0.001		0.188	1	0.6	0.002
0.188	2	0.6	0.002		0.188	2	6.5	0.030
0.188	3	0.9	0.003		0.188	3	6.1	0.029
0.188	4	2.2	0.005		0.188	4	6.2	0.032
0.188	5	0.8	0.006		0.188	5	5.3	0.032
0.188	6	1.4	0.007		0.188	6	5.7	0.022
0.188	7	0.5	0.001		0.188	7	2.6	0.005
0.188	8	0.5	0.001		0.188	8	5.5	0.025
ave		**0.9**	**0.003**		**ave**		**4.8**	**0.022**
0.375	1	0.8	0.004		0.375	1	mv	Mv
0.375	2	0.5	0.001		0.375	2	0.8	0.004
0.375	3	1.2	0.004		0.375	3	3.2	0.006
0.375	4	0.5	0.001		0.375	4	3.1	0.011
0.375	5	1.0	0.002		0.375	5	4.8	0.011
0.375	6	0.4	0.001		0.375	6	4.8	0.014
0.375	7	0.3	0.001		0.375	7	2.8	0.007
0.375	8	0.4	0.001		0.375	8	3.7	0.020
ave		**0.6**	**0.002**		**ave**		**3.3**	**0.010**
0.75	1	0.1	0.001		0.75	1	0.4	0.001
0.75	2	0.4	0.001		0.75	2	2.1	0.004
0.75	3	0.3	0.001		0.75	3	0.7	0.001
0.75	4	0.2	0.001		0.75	4	0.5	0.001
0.75	5	mv	mv		0.75	5	1.9	0.006
0.75	6	0.3	0.001		0.75	6	0.4	0.001
0.75	7	0.4	0.001		0.75	7	2.2	0.007
0.75	8	0.5	0.001		0.75	8	0.2	0.001
ave		**0.3**	**0.001**		**ave**		**1.1**	**0.003**
1.5	1	0.2	0.001		1.5	1	0.2	0.001
1.5	2	0.4	0.001		1.5	2	0.5	0.001
1.5	3	0.3	0.001		1.5	3	0.3	0.001
1.5	4	0.6	0.001		1.5	4	0.6	0.001
1.5	5	0.2	0.001		1.5	5	0.4	0.001
1.5	6	0.3	0.001		1.5	6	0.3	0.001
1.5	7	0.3	0.001		1.5	7	0.6	0.002
1.5	8	0.4	0.001		1.5	8	0.3	0.001
ave		**0.3**	**0.001**		**ave**		**0.4**	**0.001**
3	1	0.1	0.001		3	1	0.2	0.001
3	2	0.4	0.001		3	2	0.3	0.001
3	3	mv	mv		3	3	0.6	0.001

3	4	0.3	0.001		3	4	0.4	0.001
3	5	1.1	0.002		3	5	0.6	0.002
3	6	0.5	0.001		3	6	0.3	0.001
3	7	0.4	0.001		3	7	0.3	0.001
3	8	0.2	0.001		3	8	0.4	0.001
ave		**0.4**	**0.001**		**ave**		**0.4**	**0.001**

Results initially subjected to ANOVA (after $\log_e$ transformation), significant (Fpr < 0.001) difference between biotypes. Data then subjected to non-linear regression analysis with lack-of-fit test.

LD_{50}'s (de-transformed - mg a.i./litre, standard errors in parenthesis)

Clev (R) = 0.36 (0.06), Halja (S) = 0.101 (0.02)

Resistance index = 3.6

Fresh weight of new shoot growth of trimmed seedlings of Italian rye-grass at the 2-3 leaf stage treated with isoproturon.