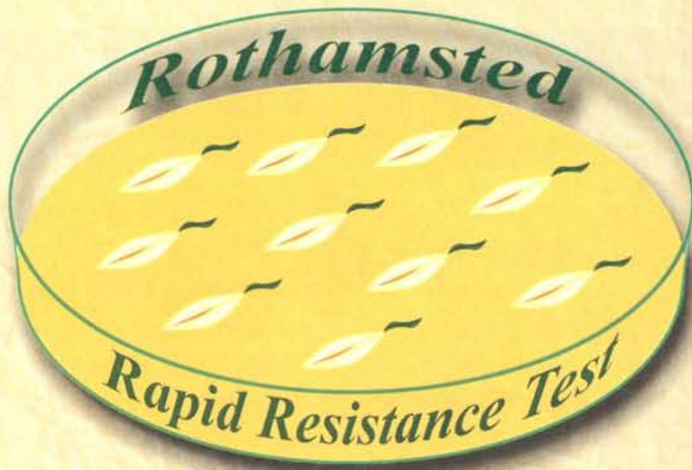

For detecting herbicide-resistance in Black-grass Wild-oats & Italian rye-grass

by Stephen Moss IACR-Rothamsted

Rothamsted Rapid Resistance Test

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Summary

This test detects resistance to herbicides in the grass-weeds black-grass (*Alopecurus myosuroides*), wild-oats (*Avena* spp.) and Italian rye-grass (*Lolium multiflorum*). Seeds are germinated in Petri-dishes containing filter papers soaked with specific concentrations of herbicide: fenoxaprop-P-ethyl (10 ppm), sethoxydim (10 ppm) and pendimethalin (5 ppm) for black-grass; fenoxaprop-P-ethyl (7.5 ppm) for wild-oats; diclofop-methyl (7.5 ppm) for Italian rye-grass. Control dishes contain potassium nitrate solution only. The number of seeds with shoots over 1 cm long is determined after 2 weeks and this provides a measure of the degree of resistance. A key element is the inclusion of standard reference populations (both resistant and susceptible) which greatly aid the interpretation of the results. Although this test requires a seed sample, it is much faster, and potentially cheaper, than the current standard glasshouse pot test - the aim is to obtain a result by mid-late September for seed samples collected in July. The test also provides an indication of the resistance mechanisms present - this information can help in planning the best herbicide strategy to use in the field.

Protocol

Although the test is relatively simple and unsophisticated, attention to detail is vital. There are six main elements to the test:

1. ***Seed collection.***
2. ***Seed cleaning and dormancy breaking storage treatment.***
3. ***Preparing Petri-dishes.***
4. ***Preparing and adding herbicides to dishes.***
5. ***Assessing Petri-dishes.***
6. ***Interpretation of results.***

These are dealt with in turn. The technique has been developed mainly with black-grass. Details for the other grasses are also presented; these might need modifying in the light of further studies. The key aspects are highlighted in **bold text**.

Black-grass (Alopecurus myosuroides)

Wild-oats (Avena spp.)

Italian rye-grass (Lolium multiflorum)

Black-grass:

In winter cereals the **best time to collect seeds is usually the second and third week in July** when about 10-20% seeds have already shed. Even in early years, June is too early. In spring sown crops (e.g.linseed), seedcollection may need to wait until August.

Wild-oats:

In winter cereals, **the second half of July or early August** is usually best.

Italian rye-grass:

Timing is more difficult to predict as seeds tend to shed rapidly, but **early to mid July** is usually optimal.

Adequate *quantities* of seeds must be collected. The equivalent of a mugfull is ideal for black-grass.

- **Collect ripe seeds of black-grass and Italian rye-grass by gently rubbing heads over a small polythene bag. Wild-oats are most easily collected by holding panicles inside a large polythene bag and shaking vigorously**. Samples collected in the proper manner will consist almost entirely of seeds, with no stalks, chaff or other debris.

- **Aim to collect over an area of about 100 m by 2-3 tramlines** within the main problem area, unless the problem is confined to a smaller very distinct patch. Avoid obviously unsprayed areas. The sample needs to be representative of the problem field or area, so a few seeds from lots of heads should be collected. Make a sketch map of area sampled.

- Quality is more important than quantity. Only a few hundred seeds are required for the test, but **aim to collect at least a mugfull of seeds** to allow for losses during drying and cleaning. This should take less than 15 minutes.

- **Do not strip heads by pulling off all seeds** - many will not be viable if they are not easily detached from the head.

Seed samples should be clean and free from debris. Neither of these wild-oat samples has been cleaned, but that on the left was collected in the correct manner, that on the right was not.

- **Do not simply collect heads from the field** - this just makes more work subsequently and usually results in a poor quality seed sample.

- **Do not collect in very wet conditions** - seeds can become very dormant.

- **Beware of rapid heating of freshly collected samples** - do not store in polythene bags.

Seeds are best collected in polythene bags in the field and then immediately transferred to paper envelopes for transport and storage. (**Practical tip**: Use envelopes with end (not side) flaps. Staple side and bottom seams to prevent them coming unstuck due to moisture from seeds). Label envelope with name of field, farm and date of collection. Air dry seeds as soon as possible after collection. Small samples can be dried in the envelopes by simply standing them on end with the flap open and shaking the envelope daily. Larger samples are best dried in trays placed in a dry, well ventilated, but not windy, environment. Seeds should be dry within about a week.

Tests for resistance require seed samples which must be of good *quality*. Polythene bags are good for seed collection purposes but do not *store* seeds in them.

2. Seed cleaning and dormancy breaking storage treatment.

However good the sampling, some of the seeds collected will be "empty" (no caryopsis) and thus be incapable of germination. Most freshly collected seeds of black-grass and wild-oats will also be dormant. The aim of cleaning and the storage treatment is to maximise the potential germination capacity of the seeds.

- **As soon as possible after seed samples have been air dried, (from about 1 week after collection), remove "empty seeds" from black-grass samples preferably using an air column seed separator. Alternatively passing seeds in front of a fan (hair drier? fan heater on cold setting?), or blowing on seeds in a tray can help remove most of the lighter, empty seeds.**

- **Cleaning is absolutely essential with black-grass samples** as it increases the proportion of viable seeds in the sample considerably. Usually there is no need to clean wild-oat and Italian rye-grass seed samples, apart from picking out any bits of stem or panicle.

- A good indication of the proportion of viable seeds in a sample of **black-grass** can be obtained by counting and weighing sub-samples of 100 seeds, and calculating the numbers of seeds/g: <400 seeds/g = >85% viability, excellent; 500 seeds/g = 70 % viability, good; 700 seeds/g = 45% viability, mediocre; 900 seeds/g = 20% viability, poor. Most wild-oat and Italian rye-grass seed should be viable if collected as described above. Dissecting seeds of all three species to see if a caryopsis is present can also be a useful method of establishing the quality of the sample.

- **As soon as the black-grass and wild-oat seeds have been air dried and cleaned, transfer to an incubator set at a constant temperature in the range 30-35 °C. Seeds need to be kept at 30-35 ° C for at least 2 weeks, and preferably 4 weeks or more, to maximize the reduction in seed dormancy.** High temperature storage helps break dormancy in black-grass and wild-oats, probably in the same way as drought stressed plants tend to produce less dormant seed. The seeds are best stored in labelled and sealed paper envelopes which will allow any residual moisture to disperse.

(**Practical tip**: small self-seal 10 x 10 cm "pay packet" type envelopes are ideal). In the absence of a dedicated incubator, alternative improvisations may be suitable. The aim is to achieve low humidity but a relatively high temperature, preferably over 30 ° C (but less than 50 ° C), (roof of barn? sunny window sill? above central heating boiler? airing cupboard?).

- Italian rye-grass seeds tend to have less dormancy so there is no need for high temperature storage. Storing seeds in envelopes at 15-20 °C is the best policy.

- Keep seeds dry and protected from vermin and disturbance.

The quality of black-grass samples can be greatly increased by the use of an air column seed cleaner

Black-grass (8 dishes per population)

- **Mix each seed sample thoroughly, and then for each population, place exactly 50 seeds in each of 8 Petri-dishes (9cm) containing four filter papers (three cellulose filter papers (Whatman No 1) and one glass-fibre filter paper (Whatman GF/A) on top). Dishes must be the correct way up (Fig. 1) otherwise paper dries out!** The filter papers do not fit neatly into the dishes so it is necessary to run your finger nail around the inner circumference of the dish to ensure the papers sit reasonably flat.

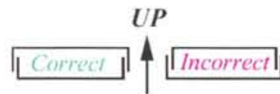


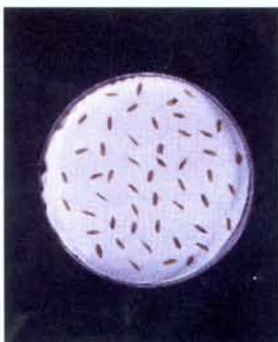
Fig. 1

> **Practical tip**: For counting out seeds use paper with vertical lines drawn at about 2 cm spacings, spread a pinch of seeds over the sheet and count up and down the columns. Add or remove seeds as necessary and then tip sample of 50 seeds into the Petri-dish.

- **Label the lid of each dish with its population name using a permanent felt tip marker. In addition, for each set of 8 dishes, label two "NIL", two "FEN", two "SETH", and two "PEND". For each pair, label one "rep I", the other "rep II".**

- Prepare all dishes before starting to add herbicide solutions. **Note that preparation of dishes containing dry seeds can be done at any time after seed samples are cleaned. It may be convenient to count seeds into dishes immediately after cleaning and then store the dishes at 30-35 ° C, rather than using envelopes.**

- **Include standard reference populations (at the very least a susceptible standard) in every test.** See interpretation of results section for more details of these.



A Petri-dish containing 50 black-grass seeds

A Petri-dish containing 50 wild-oat seeds

Wild-oats (4 dishes per population)

- A similar system to black-grass is used - refer to details above. **Place 50 seeds in each of 4 Petri-dishes.** For samples of winter wild-oats (*Avena ludoviciana*), individual seeds need to be obtained by "splitting", as the dispersal unit is a spikelet containing 2-3 seeds. Discard small 3rd seeds. With *Avena fatua*, seeds are shed individually so "splitting" is not necessary. **Label the lid of each dish with the population name, and label two dishes "NIL" and two "FEN". For each pair, label one "rep I", the other "rep II".**

- **To ensure maximum germination it is essential that wild oat seeds are pricked with a mounted needle.** This is best done immediately prior to adding herbicide solutions, but alternatively can be done any time after seed cleaning. Simply prick all the seeds in each dish through the middle with a coarse needle. It is easily possible to prick over 500 seeds per hour. Do not prick too near the base of the seed (damage to the embryo) or too near the tip (ineffective). The aim is to prick the caryopsis, but not the embryo.

Italian rye-grass (4 dishes per population)

- A similar system to black-grass is used - refer to details above. **Place 50 seeds in each of 4 Petri dishes. Label the lid of each dish with the population name, and label two dishes "NIL" and two "DIC". For each pair, label one "rep I", the other "rep II".**

4. Preparing and adding herbicides to dishes.

Herbicides must only be handled by people trained in their safe use and disposal. Appropriate protective clothing, including face shield and gloves, must be worn when handling concentrated herbicide. The diluted herbicide should pose much less risk as the concentrations used on the filter papers represent less than 3% of the typical concentrations in sprayers when applying these herbicides in the field. (See 'Safety Information' on opposite page)

The resistance test is best conducted in an incubator with lights

Black-grass

The details below are appropriate for treating up to 17 populations (8 dishes per population). If more than this number are to be treated, simply scale up amounts accordingly (there is no need to scale up herbicide stock solutions).

- **Prepare a 1 litre solution of potassium nitrate in distilled or deionised water (2 g/litre).** The use of potassium nitrate is not essential but it does help break seed dormancy and promote seedling growth. Do not be tempted to use higher concentrations as these tend to be toxic. Tap water is probably a suitable substitute for distilled water if this is unavailable.

Fenoxaprop (FEN).

- **Half fill a 250 ml measuring cylinder with distilled water, then measure out as accurately as possible 5.7 ml of a product containing 55 g fenoxaprop-P-ethyl/litre using a 10 ml syringe, add this to the cylinder and make up to 250 ml with more water to form a stock solution of fenoxaprop-P-ethyl. Mix thoroughly and label.**

  Half fill a second 250 ml measuring cylinder with potassium nitrate solution, then measure out exactly 2 ml of the fenoxaprop stock solution using a 2 ml syringe, add to cylinder and make up to 250 ml with more potassium nitrate solution. Mix thoroughly and label. This solution contains 10 ppm fenoxaprop-P-ethyl.

  (This double dilution method is used as it is not easy to directly measure out the tiny quantities of herbicides needed - in the above case 0.045 ml of product).

- Before adding herbicide solution to dishes, ensure seeds are reasonably evenly spaced over the surface of the filter paper - not all at one side. **Add 7 ml of the 10 ppm fenoxaprop solution to the filter papers in each of the "FEN" labelled Petri-dishes.** This can be done using a 10 ml syringe, or for large number of dishes, an adjustable dispenser. Replace Petri-dish lids.

  (The 7 ml added to each dish does not need to be too exact as it is the herbicide concentration that is important, rather than the amount. This amount of herbicide will soak the four filter papers without leaving any free liquid. Certainly the amount applied should not vary between dishes by more than ± 0.5 ml.)

Sethoxydim (SETH).

The basic procedure is the same as for fenoxaprop. **Take 1.62 ml of a product containing 193 g sethoxydim/litre using a 2 ml syringe to make up the 250 ml stock solution. Use 2 ml of this stock solution in 250 ml potassium nitrate to produce a 10 ppm sethoxydim solution. Add 7 ml of the 10 ppm sethoxydim solution to the filter papers in each of the "SETH" labelled Petri-dishes.**

Pendimethalin (PEND).

The basic procedure is the same as for fenoxaprop. **Take 0.78 ml of a product containing 400 g pendimethalin/litre using a 1 ml syringe to make up the 250 ml stock solution. Use 1 ml (not 2 ml) of this stock solution in 250 ml potassium nitrate to produce a 5 ppm pendimethalin solution. Add 7 ml of the 5 ppm pendimethalin solution to the filter papers in each of the "PEND" labelled Petri-dishes.**

Controls (NILS).

Add 7 ml of the potassium nitrate solution to the filter papers in each of the "NIL" labelled Petri-dishes.

Ideally all three herbicides should be used in each test. However, the use of sethoxydim alone will allow the detection of target site resistance. This may be useful where enhanced metabolism is already known to exist. Sethoxydim works particularly well in Petri-dishes and should give clear results even in sub-optimal conditions.

IMPORTANT SAFETY INFORMATION

The test requires the use of formulated herbicide products. Various statutes and regulations[1] govern the use of pesticides and other chemicals. Users should make themselves aware of the hazards associated with using pesticides and other chemicals, and have in place the controls to minimise risk of exposure. Provision of appropriate protective clothing and proper means of disposal of unused pesticides and other chemicals must be considered before preparing and adding herbicides to dishes.

[1] Health & Safety at Work Act 1974, Food & Environmental Protection Act 1985, Control of Substances Hazardous to Health Regulations 1994, Environmental Protection (Duty of Care) Regulations 1991, Control of Pesticides Regulations 1986, Control of Pesticides (Amendment) Regulations 1997.

Place dishes in clear polythene bags (Practical tip: the sleeves the Petri-dishes come in are ideal.) **Keep treatments and replicates separate** i.e. stack all "FEN I" dishes together in one sleeve, all "FEN II" dishes in another sleeve, all "NIL I" dishes in another sleeve etc. (This minimizes any cross contamination in the vapour phase between treatments). Up to 20 dishes can be placed in one sleeve. If more than this number are needed, split equally between two sleeves. **Place an empty Petri-dish containing filter papers on the top of each stack so that the top dish receives the same amount of light as the rest, and seal the top of the sleeve with a rubber band or tape.**

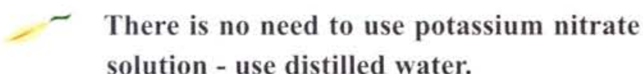
Place stacks of dishes in an incubator set to provide a 17°C, 14 hour day (lights on) and a 10°C, 10 hour night (lights off). In the absence of an incubator, place stacks where they will receive some indirect light (needed for germination) but do not place in direct sunlight otherwise temperature within the dishes is likely to be excessive. Aim to maintain the temperature range within 10-20 °C. Avoid temperatures over 25° C.

Move stacks around within incubator every 1-2 days to maintain as uniform an environment as possible across all treatments.

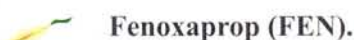
Petri-dishes should be ready for assessing after 2 weeks.

Wild-oats

The same procedure is used as described for black-grass, with the following differences.

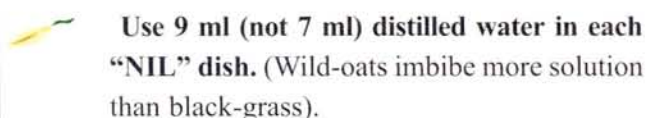


There is no need to use potassium nitrate solution - use distilled water.



Fenoxaprop (FEN).

Use **5.7 ml of a product containing 55 g fenoxaprop-P-ethyl/litre using a 10 ml syringe to make up the 250 ml stock solution (same as for black-grass). Use 1.5 ml (not 2 ml) of stock solution in 250 ml distilled water to produce a 7.5 ppm fenoxaprop solution. Add 9 ml (not 7 ml) of the 7.5 ppm fenoxaprop solution to the filter papers in each of the "FEN" labelled Petri-dishes.**

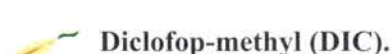


Use 9 ml (not 7 ml) distilled water in each "NIL" dish. (Wild-oats imbibe more solution than black-grass).

Italian rye-grass

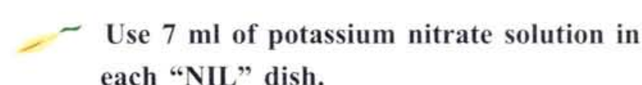
The same procedure is used as described for black-grass, with the following differences.

Use potassium nitrate solution as described.



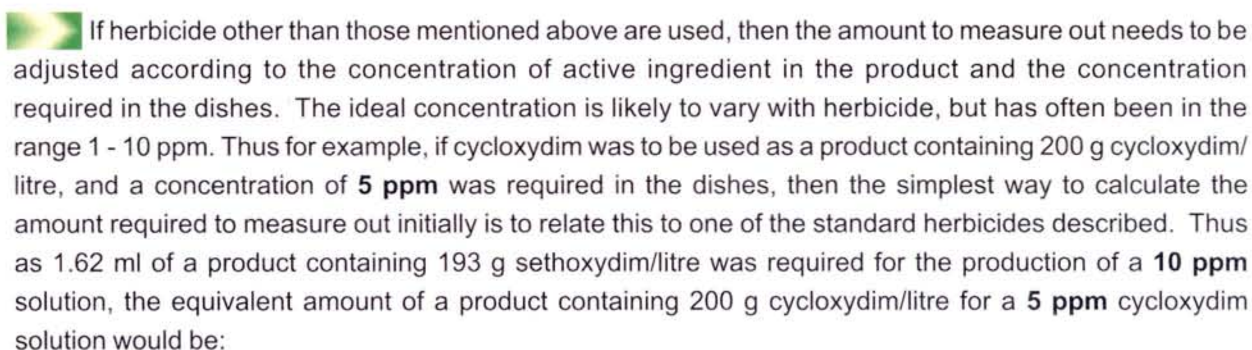
Diclofop-methyl (DIC).

Use **0.83 ml of a product containing 378 g diclofop-methyl/litre using a 1 ml syringe to make up the 250 ml stock solution. Use 1.5 ml (not 2 ml) of stock solution in 250 ml potassium nitrate to produce a 7.5 ppm diclofop solution. Add 7 ml of the 7.5 ppm diclofop solution to the filter papers in each of the "DIC" labelled Petri-dishes.**



Use 7 ml of potassium nitrate solution in each "NIL" dish.

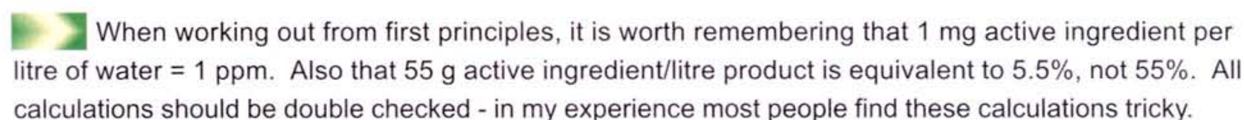
Notes:



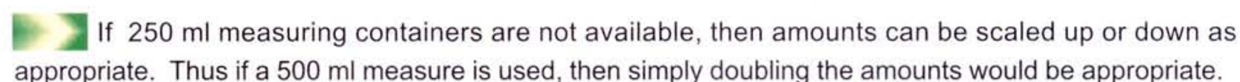
If herbicide other than those mentioned above are used, then the amount to measure out needs to be adjusted according to the concentration of active ingredient in the product and the concentration required in the dishes. The ideal concentration is likely to vary with herbicide, but has often been in the range 1 - 10 ppm. Thus for example, if cycloxydim was to be used as a product containing 200 g cycloxydim/litre, and a concentration of **5 ppm** was required in the dishes, then the simplest way to calculate the amount required to measure out initially is to relate this to one of the standard herbicides described. Thus as 1.62 ml of a product containing 193 g sethoxydim/litre was required for the production of a **10 ppm** solution, the equivalent amount of a product containing 200 g cycloxydim/litre for a **5 ppm** cycloxydim solution would be:

$$[(5 \text{ ppm} \div 10 \text{ ppm}) \times 1.62 \text{ ml} \times (193\text{g/l} \div 200\text{g/l})] = 0.78 \text{ ml product}$$

Note: the **more** concentrated the herbicide formulation, the **less** that is required



When working out from first principles, it is worth remembering that 1 mg active ingredient per litre of water = 1 ppm. Also that 55 g active ingredient/litre product is equivalent to 5.5%, not 55%. All calculations should be double checked - in my experience most people find these calculations tricky.



If 250 ml measuring containers are not available, then amounts can be scaled up or down as appropriate. Thus if a 500 ml measure is used, then simply doubling the amounts would be appropriate.

5. Assessing Petri-dishes.

- Assess dishes 2 weeks after adding herbicides.Use the assessment sheets provided in this protocol. (They can be photocopied.)

- Count and record the number of seeds which have germinated in each NIL (control) dish. "Germinated" means that an emerging root or shoot is visible. There is **no** need to assess number of seeds germinated in dishes containing herbicide.

- For all NIL **and** herbicide treatments, count and record the number of shoots in each dish which are 1 cm or more in length. There is no need to actually measure these as the distance from the filter papers to the lid of the Petri-dish is 1 cm. So what you actually count is the number of shoots "hitting the lid" (including those which would have" hit the lid"had they been growing vertically).

Treated dish (sethoxydim) after 2 weeks - note extensive seedling growth indicating resistance

- For the assessments of germination (NILS only) and numbers of shoots over 1 cm (NILS and all herbicide treatments), add the two replicate values together, which will give a % figure (as there were 50 seeds in each of two dishes). The % germination in the NIL dishes should ideally be over 75% - the higher the better.

- Calculate the % of germinated seeds which have shoots over 1 cm for the NIL dishes. (e.g. If numbers of seeds germinated is 75 (total of two NIL dishes) and number of seeds with shoots over 1 cm is 60 (total of two NIL dishes), the % of germinated seeds with shoots over 1 cm will be [(60 ÷ 75) x 100] = 80%. The higher this figure is for NIL dishes the better. Typically it should be 80-90%. If it is less than 70%, it may be best to leave dishes for a further week and re-assess them.

- For all populations and treatments, calculate the % reduction in shoots over 1 cm relative to the NIL values for the same population. (e.g. If the number of seeds with shoots over 1 cm in NIL dishes is 60 (total of two dishes), and number of seeds with shoots over 1 cm is 20 for the "FEN" treatment for the same population (total of two dishes), then % reduction is [(60 - 20) ÷ 60] x 100 = 67%. If no seeds have produced shoots over 1 cm, then the % reduction for that treatment will be 100%. Conversely if a herbicide treatment has had no effect and the same number of seeds have produced shoots over 1 cm long in both the NIL and treated dishes, then % reduction will be 0%. Negative % reductions are possible too!

- The susceptible standard populations should have high % reduction values (preferably over 90%) and the resistant standard populations should have low % reduction values (preferably less than 50%).

- If conditions for germination and shoot growth have not been ideal, it may be better to assess 3 weeks after adding herbicides.

Treated dish (fenoxaprop) after 2 weeks - note absence of growth indicating susceptibility.

Examples of results and calculations (actual results)

NIL dishes (50 seeds per dish)

Population	Number of germinated seeds		%	Number of seeds with shoots >1 cm		*	% of germinated seeds with shoots >1 cm
	Dish I	Dish II	Total	Dish I	Dish II	Total	
ROTH	46	44	90	38	41	**79**	[(79 ÷ 90) x 100] = 88%
PELD	44	49	93	41	41	**82**	[(82 ÷ 93) x 100] = 88%
OXON	41	38	79	29	28	**57**	[(57 ÷ 79) x 100] = 72%
LINCS	43	43	86	40	39	**79**	[(79 ÷ 86) x 100] = 92%

Germination % was excellent for ROTH and PELD, and good for the other two populations. The % of germinated seeds with shoots > 1 cm was also good for three populations (≥88%) and satisfactory for OXON at 72%. Had it been substantially lower than 70% it might have been best to wait for up to another week before assessing.

Fenoxaprop (FEN) (50 seeds per dish)

Population	Number of seeds with shoots >1 cm			% reduction in number of seeds with shoots >1cm relative to NILS
	Dish I	Dish II	Total	
ROTH	2	1	3	[(79 - 3) ÷ 79] x 100 = **96%**
PELD	15	28	43	[(82 - 43) ÷ 82] x 100 = **48%**
OXON	25	26	51	[(57 - 51) ÷ 57] x 100 = **11%**
LINCS	40	30	70	[(79 - 70) ÷ 79] x 100 = **11%**

Note: The appropriate NIL value to use in these calculations is the total (of two dishes) **number** of seeds with shoots > 1 cm for that population (* column in previous table), **not the %** of germinated seeds with shoots > 1 cm.

Control of ROTH (a susceptible standard) was excellent. Control of PELD was mediocre (it is a standard for enhanced metabolism based resistance). Control of the other two populations was very poor - in OXON because virtually the whole population has target site resistance to 'fops' and 'dims' and in LINCS because of multiple mechanisms (enhanced metabolism, target site and other as yet uncharacterised mechanisms.)

Sethoxydim (SETH) (50 seeds per dish)

Population	Number of seeds with shoots >1 cm			% reduction in number of seeds with shoots >1cm relative to NILS
	Dish I	Dish II	Total	
ROTH	0	0	0	[(79 - 0) ÷ 79] x 100 = **100%**
PELD	0	0	0	[(82 - 0) ÷ 82] x 100 = **100%**
OXON	36	28	64	[(57 - 64) ÷ 57] x 100 = **-12%**
LINCS	7	4	11	[(79 - 11) ÷ 79] x 100 = **86%**

Note: The appropriate NIL value to use in these calculations is the total (of two dishes) **number** of seeds with shoots > 1 cm for that population (* column in table for NILS), **not the %** of germinated seeds with shoots > 1 cm. Also note (for OXON) that negative % reduction values are possible.

Control of ROTH (a susceptible standard) was excellent. Control of PELD was also excellent as enhanced metabolism based resistance does not appear to affect sethoxydim. Control of OXON was very poor because of the presence of target site resistance to 'fops' and 'dims'. In LINCS control was reasonable as sethoxydim appears to be only vulnerable to target site resistance, and not to the other mechanisms that can affect fenoxaprop. In LINCS we know that only about 10% of the population has target site resistance, and so overall level of control by sethoxydim was reasonably good.

Pendimethalin (PEND) (50 seeds per dish)

Population	Number of seeds with shoots >1 cm			% reduction in number of seeds with shoots >1cm relative to NILS
	Dish I	Dish II	Total	
ROTH	1	0	1	[(79 - 1) ÷ 79] x 100 = **99%**
PELD	30	30	60	[(82 - 60) ÷ 82] x 100 = **27%**
OXON	5	5	10	[(57 - 10) ÷ 57] x 100 = **83%**
LINCS	8	12	20	[(79 - 20) ÷ 79] x 100 = **75%**

Note: The appropriate NIL value to use in these calculations is the total (of two dishes) **number** of seeds with shoots > 1 cm for that population (* column in table for NILS), **not the %** of germinated seeds with shoots > 1 cm. Also note (for OXON) that negative % reduction values are possible.

Control of ROTH (a susceptible standard) was excellent. Control of PELD was poor due to presence of the enhanced metabolism mechanism. Control of OXON and LINCS was mediocre due to low levels of enhanced metabolism in both populations.

6. Interpretation of the results (for black-grass).

- The values for the % reduction in number of seeds with shoots > 1 cm relative to NILS are calculated as described above for all populations and tabulated.

- The interpretation of the results is greatly helped by the inclusion of standard populations with known resistance characteristics. Results for four standard populations are given in the examples in the previous section. Details of these standards are:

ROTH: (Rothamsted; susceptible standard) susceptible to all herbicides.

PELD: (Peldon; enhanced metabolism standard) shows resistance to a wide range of different herbicides due to the enhanced metabolism mechanism. No target site resistance in this population.

OXON: (target site standard) shows a high level of resistance to all 'fops' and 'dims' due to target site resistance. In addition shows a low level of enhanced metabolism - much less than Peldon.

LINCS: (multiple resistance mechanisms standard) shows a high level of resistance to several 'fop' and 'dim' herbicides due to the presence of multiple mechanisms including enhanced metabolism, target site resistance in about 5-10% of the population and additional unknown mechanisms. This population also shows partial resistance to some other herbicides (e.g. isoproturon, pendimethalin) due to a low level of enhanced metabolism.

- **The inclusion of a susceptible standard population such as Rothamsted is essential**, and the inclusion of other standard reference populations is highly desirable. A high level of control of the susceptible standard should be achieved, otherwise the results for other populations will be of dubious validity. The concentrations recommended in this protocol should give over 90% reduction for the susceptible standard. If less than 80% reduction is achieved with any herbicide, treat all results for that herbicide with caution.

- **Fenoxaprop, sethoxydim and pendimethalin** are used for the following reasons:

Fenoxaprop is used to indicate resistance to this herbicide and likely resistance to other 'fops' and tralkoxydim. Fenoxaprop and other 'fops' are vulnerable to both enhanced metabolism, target site resistance and other, as yet uncharacterised mechanisms. Thus it is not possible to deduce which mechanisms are responsible solely from the fenoxaprop result.

Sethoxydim is used to indicate **target site resistance** which gives virtually complete resistance to **all** 'fops' and 'dims' (sethoxydim and cycloxydim are not affected by enhanced metabolism, unlike most other 'fops' and 'dims').

Pendimethalin is used to indicate **enhanced metabolism** affecting performance, not only of pendimethalin, but also chlorotoluron, isoproturon, flupyrsulfuron and many other herbicides.

N.B. Several different mechanisms can occur within the same population.

Fenoxaprop results

The lower the % reduction value, the greater the degree of resistance and hence the risk of inadequate control from this, and similar herbicides. Resistance to fenoxaprop is also likely to extend to: clodinafop, diclofop , other 'fops' to some degree, and tralkoxydim. Clodinafop tends to be less affected (but not unaffected) by resistance than fenoxaprop. Fenoxaprop resistance will not necessarily affect performance of the 'dims' sethoxydim and cycloxydim.

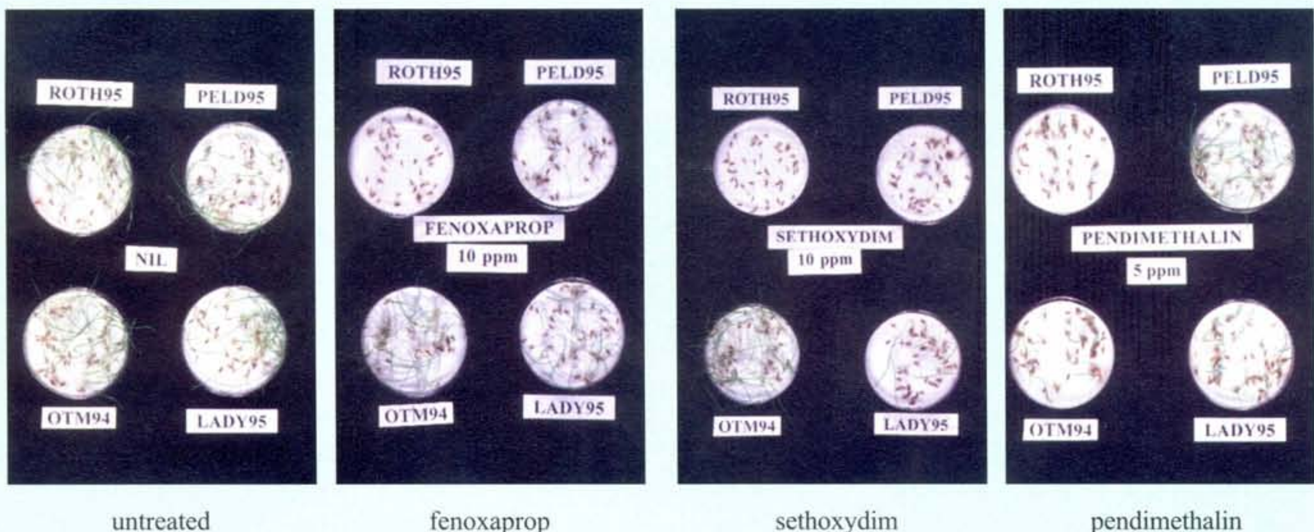
Sethoxydim results

The lower the % value, the more severe the level of target site resistance, and hence the higher the probability of complete failure of all 'fop' and 'dim' herbicides to control resistant black-grass. Even a low level of target site resistance should be cause for concern, as this type of resistance has the potential to increase rapidly. Minimize use of all 'fops' and 'dims'.

Pendimethalin

The lower the % value, the greater the resistance due to enhanced metabolism and hence the greater the chance of reduced activity from many herbicides, including not only pendimethalin, but also isoproturon and flupyrsulfuron. Bear in mind that resistance due to enhanced metabolism is rarely absolute, and that even at Peldon (which has a high level of enhanced metabolism) herbicides such as isoproturon still give useful, if reduced, levels of control.

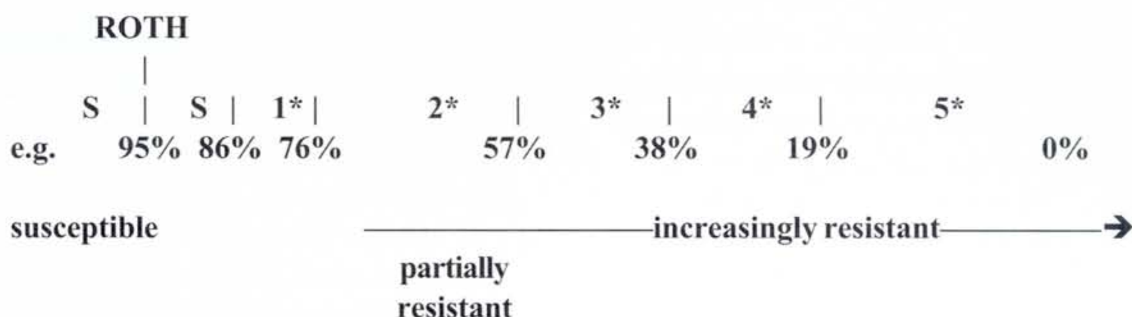
When resistance is absolute, interpretation is relatively easy. More commonly resistance is partial, making interpretation more difficult. A modified * rating system is recommended as a means of dealing with this problem. The latest version of the * rating system requires the inclusion of just a single standard reference populations in every test - ROTH (susceptible). Previous versions have required the inclusion of two or three standards. 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 if desired and utilises the same susceptible standard (ROTH) for all herbicides.



untreated | fenoxaprop | sethoxydim | pendimethalin

The % reduction values between ROTH and zero are separated into **five** equal categories (see diagram). One of these categories, at the ROTH end of the range, is subdivided about its mid point into two smaller categories, S and 1*. In addition 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 (ROTH) population in each individual test. The actual values delineating the categories will differ between tests.

Thus if the ROTH % reduction value was 95%, each category would be 19% (i.e. 95% ÷ 5 = 19%). Thus <19% = 5*; 19% to 38% = 4*; 38% to 57% = 3*; 57% to 76% = 2*; 76% to 85.5% = 1*; 85.5% to 95% (and over) = S (susceptible).



The higher the * rating the greater the degree of resistance.

- In practice, the six categories calculated above are more than are needed for screening purposes, so the following four category system is suggested: 5*/4* = RRR (resistant); 3*/2* = RR (partially resistant); 1* = R? (marginal insensitivity); S = S (susceptible). The reference to 1* as R? is probably appropriate as field experiments show that in certain conditions populations rated as 1* may result in reduced herbicide efficacy. The 1* (R?) category for sethoxydim should be taken as positive evidence of target site resistance, albeit at a relatively low frequency.

- The resistance test only relates to the sample submitted. Obvious but important. How representative this is of the entire field depends on the method of sampling and the proportion of plants which survived treatment in the field. Thus, if the seed sample was collected from a few surviving resistant plants, after the majority of susceptible plants were killed, the results of the test would overstate the degree of resistance present in the entire field population. This should not be viewed as a limitation but a positive attribute, as it enables resistance to be detected at an early stage of development, when it is easier to take action to prevent the situation getting worse.

- Further information on dealing with resistance grass-weeds is contained in the leaflet: *Revised Guidelines for Preventing and Managing Herbicide-Resistant Grass-weeds*, produced by the *Weed Resistance Action Group (WRAG)* with *HGCA* financial support. Copies are available free from *HGCA* or *BAA*.

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Whilst this protocol has been based on a substantial amount of research, neither the author, nor IACR-Rothamsted, nor the Ministry of Agriculture, Fisheries and Food shall in any event be liable for any loss, damage or injury howsoever suffered directly or indirectly in relation to the use of this protocol for detecting herbicide resistance.

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ASSESSMENT SHEET

Name:
Date:
Centre:

NIL Dishes (50 seeds per dish)

Population	Number of germinated seeds			Number of seeds with shoots >1cm		*	% of germinated seeds with shoots >1cm
	Dish I	Dish II	Total	Dish I	Dish II	Total	

ASSESSMENT SHEET

Name:
Date:
Centre:

Herbicide: ____________ (50 seeds per dish)

Population	Number of seeds with shoots >1cm			% reduction in number of seeds with shoots >1cm relative to NILS
	Dish I	Dish II	Total	

The appropriate NIL value to use in these calculations is the total (of two dishes) **number** of seeds with shoots > 1cm for that population, (in * column on 'NILS' assessment sheet), **not the %** of germinated seeds with shoots > 1cm

Rothamsted Rapid Resistance Test

Summary of the protocol for testing black-grass *(Alopecurus myosuroides)*.

(Refer to the full protocol leaflet for detailed instructions.)

1. Seed collection

☐ It is essential that both the **quality** and **quantity** of the seed sample is adequate. In winter cereals the best time to collect seeds is usually the **second and third week in July.** Collect the **equivalent of a mugfull** of ripe seeds by gently rubbing heads over a small polythene bag. Do not store seeds in polythene bags. Air dry seeds as soon as possible.

2. Seed cleaning and dormancy breaking storage treatment

☐ When seeds are dry, remove "empty seeds" preferably using an air column seed separator. Cleaning is absolutely essential. After cleaning transfer packets of seeds to an incubator set at a constant 30-35 °C for at least 2 weeks, and preferably 4 weeks or more.

3. Preparing Petri-dishes

☐ Mix each seed sample thoroughly, and then for each population place exactly **50 seeds in each of 8 Petri-dishes (9cm)** containing three cellulose and one glass-fibre filter paper on top. **Label the lid of each dish** with its population name. For each set of 8 dishes, label two "NIL", two "FEN", two "SETH", and two "PEND". For each pair, label one rep I, the other rep II. Include standard reference populations.

4. Preparing and adding herbicides to dishes

Herbicides should only be handled by people trained in their safe use and disposal.

☐ **Prepare a 1 litre solution of potassium nitrate in distilled water (2 g/litre).**

Fenoxaprop (FEN)

☐ Half fill a **250 ml measuring cylinder** with **distilled water**, then measure out as accurately as possible **5.7 ml of a product containing 55 g fenoxaprop-P-ethyl/litre**, add this to the cylinder and make up to 250 ml with more water to form a stock solution of fenoxaprop-P-ethyl. Mix thoroughly and label.

☐ Half fill a **second 250 ml** measuring cylinder with **potassium nitrate** solution, then measure out exactly **2 ml of the fenoxaprop stock solution**, add to cylinder and make up to 250 ml with more potassium nitrate solution. Mix thoroughly and label. This solution contains 10 ppm fenoxaprop-P-ethyl.

☐ Add **7 ml** of the 10 ppm fenoxaprop solution to the filter papers in each of the FEN labelled Petri-dishes.

Sethoxydim (SETH)

☐ Use **1.62 ml of a product containing 193 g sethoxydim/litre** to make up the **250 ml** stock solution. Use **2 ml of this stock solution** in **250 ml potassium nitrate** to produce a 10 ppm sethoxydim solution. Add **7 ml** of the 10 ppm sethoxydim solution to the filter papers in each of the SETH labelled Petri-dishes.

Pendimethalin (PEND)

☐ Use **0.78 ml of a product containing 400 g pendimethalin/litre** to make up the **250 ml** stock solution. Use **1 ml (not 2 ml) of this stock solution** in **250 ml** potassium nitrate to produce a 5 ppm pendimethalin solution. Add **7 ml** of the 5 ppm pendimethalin solution to the filter papers in each of the PEND labelled Petri-dishes.

Controls (NILS)

☐ Add **7 ml of the potassium nitrate solution** to the filter papers in each of the NIL labelled Petri-dishes.

Place dishes in clear polythene bags. Keep herbicide treatments and replicates separate. Place an empty Petri-dish containing filter papers on the top of each stack and seal the top of the bags with a rubber band. Place stacks of dishes in an incubator set to provide a 17°C 14 hour day (light on) and a 10°C 10 hour night (light off). Move stacks around within incubator every 1-2 days.

5. **Assessing Petri-dishes** (Petri-dishes should be ready for assessing after 2 weeks.)

☐ Count and record the number of seeds which have germinated in each NIL (control) dish. "Germinated" means that an emerging root or shoot is visible.

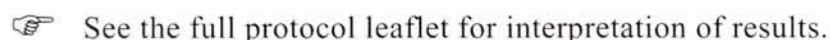
☐ For all NIL **and** herbicide treatments, count and record the number of shoots in each dish which are 1 cm or more in length (touching lid).

☐ For the assessments of germination (NILS only) and numbers of shoots over 1 cm (NILS and herbicide treatments), add the two replicate values together, to obtain a % figure.

☐ Calculate the % of germinated seeds which have shoots over 1 cm for the NIL dishes. Typically it should be 80-90%.

☐ For all populations and herbicides, calculate the % reduction in number of shoots over 1 cm relative to the NIL values for the same population (see assessment sheets).

6. **Interpretation of results**



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