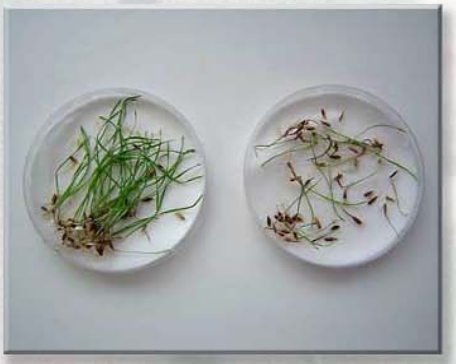
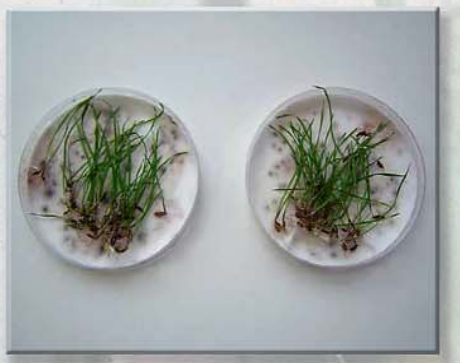


# ‘Rothamsted Rapid Resistance Test’ for ALS Inhibiting Herbicides:

## Black-grass *(Alopecurus myosuroides)*

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# ‘Rothamsted Rapid Resistance Test’ for ALS Inhibiting Herbicides: Black-grass *(Alopecurus myosuroides)*

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**Important note:** This protocol can be used as an *indicator* of ALS target site resistance using herbicides such as sulfometuron (‘Oust’) and mesosulfuron+ iodosulfuron (‘Atlantis WG’) on seed samples. Sulfometuron tends to give the most reliable results. The test is unlikely to reliably detect partial resistance due to enhanced metabolism.

## 1. Seed collection

The **quality** of the seed used in the resistance test is very important. In the UK, black-grass seeds are best collected from winter cereals during **mid July** (when approx 20% of the seeds have already shed). Seeds are best collected by gently rubbing the heads over a small polythene bag. Aim to collect the equivalent of at least a cup full of seeds. Do not store seeds in polythene bags. Air dry seeds as soon as possible and store in paper envelopes.

## 2. Seed cleaning and dormancy breaking storage treatment

When seeds are dry, remove ‘empty seeds’ preferably using an air column separator. Cleaning is essential to maximise the proportion of viable seeds. Aim to achieve a cleaned sample with 400 – 500 seeds/g, which represents a good quality sample. The viability of any sample with >600 seeds/g is likely to be inadequate to achieve a good result. Ideally, store cleaned seeds at 30-35 $^{0}$C for 2-4 weeks to break seed dormancy and consequently obtain better germination.

**IMPORTANT NOTE: The reliability of the test results is largely dependent on the quality and germination capacity of the seed sample used. Rubbish seed, rubbish results!**

## 3. Preparing Petri dishes

Mix each seed sample thoroughly, and then for each population count out exactly **50 seeds into each of six Petri-dishes (9cm) containing four filter papers** (three cellulose and one glass-fibre filter paper on top). Ensure dishes are used correct way up to avoid papers drying out – papers need to be folder around part of the circumference of each dish (Fig. 1). Label the lid of each dish with its population name. For each set of six dishes, label two ‘Nil’, two ‘SULF’ and two ‘ATL’. For each pair of dishes, label one rep 1 and the other rep 2. Always include at least one, and preferably two, susceptible reference populations. If possible, also include an ALS-resistant reference population.

**Fig 1**


## 4. Preparing and adding herbicides
(suitable for testing up to 17 populations)

Herbicides should only be handled by people trained in their safe use and disposal. Use these triple dilution methods as the herbicides are difficult to measure out directly in very small amounts.

**Prepare a 1 litre solution of 2g/litre potassium nitrate ($KNO_3$) in water** (de-ionised or distilled preferably). Use of potassium nitrate helps break seed dormancy and promotes seedling growth.

**Sulfometuron (1ppm) 'OUST' (SULF)**

Half fill a 250 ml measuring cylinder with the deionised water, and then measure out, as accurately as possible, 0.333 g of 'Oust' (containing 750 g sulfometuron/kg of product). Add this to the cylinder and make up to 250 ml with more water. Mix very thoroughly and label. This solution contains 1000 ppm sulfometuron.

Half fill a 250 ml measuring cylinder with deionised water, then measure out exactly **25 ml** of the 1000 ppm solution, add to the cylinder and make up to 250 ml with more deionised water. Mix thoroughly. This solution contains 100 ppm sulfometuron.

Finally, half fill another 250 ml measuring cylinder with potassium nitrate solution, then measure out exactly **2.5 ml** of the 100 ppm solution using a 2.5 ml syringe, add to the cylinder and make up to 250 ml with more potassium nitrate solution. Mix thoroughly. This solution contains 1 ppm sulfometuron.

Add **7ml** of the 1ppm sulfometuron solution to the filter papers in each SULF labelled Petri-dish using a syringe or dispenser.

**Mesosulfuron+Iodosulfuron (0.1 ppm) 'ATLANTIS WG' (ATL)**

Half fill a 250 ml measuring cylinder with the deionised water, and then measure out, as accurately as possible, 0.695 g of 'Atlantis WG' (containing 30 g mesosulfuron+ 6 g iodosulfuron/kg of product). Add this to the cylinder and make up to 250 ml with more water. Mix very thoroughly and label. This solution contains 100 ppm mesosulfuron+iodosulfuron.

Half fill a 250 ml measuring cylinder with deionised water, then measure out exactly **25 ml** of the 100 ppm solution, add to the cylinder and make up to 250 ml with more deionised water. Mix thoroughly. This solution contains 10 ppm mesosulfuron+iodosulfuron.

Finally, half fill another 250 ml measuring cylinder with **potassium nitrate solution**, then measure out exactly **2.5 ml** of the 10 ppm solution using a 2.5 ml syringe, add to the cylinder and make up to 250 ml with more potassium nitrate solution. Mix thoroughly. This solution contains 0.1 ppm mesosulfuron+iodosulfuron. *(For 1 ppm, use 25 ml of the 10 ppm solution)*.

Add **7ml** of the 0.1ppm mesosulfuron+iodosulfuron solution to the filter papers in each ATL labelled Petri-dish using a syringe or dispenser.

**Note:** The best single discriminating dose for mesosulfuron+iodosulfuron has usually been 0.1 ppm, but 1 ppm has sometimes given better results. It is suggested that both doses are used initially, in order to determine the best single dose to use in your specific conditions.

**Controls (NILS)**

Add **7ml** of the **$KNO_3$** solution to all filter papers in each of the NIL labelled dishes.

Place the dishes in clear polythene bags and keep the herbicides and replicates separate. Place an empty petri dish containing filter papers on the top of each stack and seal bag with a rubber band or tape. Place the stacks in an incubator set at $17^0$C, 14 hour day (with the lights on) and a $11^0$C, 10 hour night (lights off). Move the stacks around within incubator every 2-3 days.

## 5. Assessing Petri-dishes (after 2 weeks)

Measure shoot length for each germinated seed (to nearest mm) and calculate total shoot length per dish for each NIL and herbicide treated dish for all populations. Add the two replicate values together. (The individual replicate values can be used if statistical analysis is required).

For each population and herbicide, calculate the % reduction in total shoot length relative to the NIL value for the same population.

$$\% \text{ reduction} = \frac{\text{Total shoot length in NIL dishes} - \text{Total shoot length in treated dishes}}{\text{Total shoot length in NIL dishes}} \times 100$$

An alternative, and much quicker method, is to visually estimate the % reduction in shoot growth in herbicide treated, relative to NIL dishes for the same population. This correlates well with the shoot length data, but typically takes less than 5% of the time required to measure shoots. As visual estimates are more subjective, they should be performed but at least two, and preferably more, independent assessors.

## 6. Interpretation of results

It is important to recognise that at the dose used (and even at considerably higher doses), these ALS inhibiting herbicides will not prevent germination and seedling growth of susceptible black-grass in Petri-dishes. However, growth will be reduced relative to resistant populations. Consequently, this test for ALS target site resistance is not as robust as Petri-dish tests for target site resistance to ACCase inhibiting herbicides ('fops' and 'dims').

The inclusion of a susceptible reference population is essential. Ideally use two susceptible populations. The interpretation of the results is **greatly** helped by the inclusion of a reference ALS target site resistant population, in which resistance has been fully characterised at the whole plant, biochemical and molecular level.

Example of actual results for three reference and five test populations collected in 2006.

| | Mesosulfuron + iodosulfuron at 0.1 ppm | | | |
|---|---|---|---|---|
| | Measured | | Visual | |
| Population | % reduction in shoot length | 'R' ratings | % reduction in growth | 'R' ratings |
| Suscepitble standard* | 66* | S | 77* | S |
| Enhanced metabolism standard | 66 | S | 72 | S |
| ALS target site resistant standard* | 9* | RRR | 2* | RRR |
| WILTS | 67 | S | 72 | S |
| NOTTS | 53 | R? | 65 | R? |
| LINCS | 10 | RRR | 17 | RRR |
| ESSEX | 6 | RRR | 9 | RRR |
| OXON | -6 | RRR | 3 | RRR |

See pg 6 for details of 'R' rating system. S =susceptible, R?, RR and RRR indicate higher degrees of resistance. * see photos of the susceptible and ALS target site resistant standards on page 7.

The lower the % reduction values, relative to the susceptible reference population, the greater the degree of resistance and hence the risk of inadequate control from ALS inhibiting herbicides. Control of the susceptible and enhanced metabolism standards was similar, showing that the test does not detect resistance to this herbicide conferred by enhanced metabolism. Control of the ALS target site resistant standard was much poorer, indicating that the test did detect ALS target site resistance. Control of the WILTS population was similar to the susceptible standard, indicating the absence of ALS target site resistance. Control of the LINCS, ESSEX and OXON populations was very poor, and similar to the ALS target site resistant standard. This is a good indication that these populations were resistant due to ALS target site resistance, although it is possible that other, as yet unidentified, mechanisms are responsible. The NOTTS 2006 population showed intermediate results, possibly because only a relatively small proportion of the population was resistant. % reductions based on the much quicker visual assessment were mainly slightly higher than those based on shoot measurements, although the relative differences between populations were the same.

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 the susceptible plants were killed, the results 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.

The Petri-dish test is much faster and cheaper than the standard glasshouse pot test – the aim is to obtain a result by mid September for seed samples collected in July. Pot tests give more reliable results, but typically take 8 weeks to complete compared with 2 weeks for the Petri-dish test.

Other herbicides can be included to give a more comprehensive characterisation. For example, sethoxydim or cycloxydim can be used as *indicators* of ACCase target site resistance affecting both 'fops' and 'dims', and pendimethalin used as an *indicator* of enhanced metabolism resistance in black-grass. See the 'Rothamsted Rapid Resistance Test' (1999) leaflet for detailed instructions.

**'R' rating system**

This system assigns samples to four resistance categories depending on the degree of control achieved relative to a susceptible reference population in single dose screening assays. The determination of the different categories is made using the % reduction value obtained for the susceptible reference population in each individual test. The four categories are: RRR (resistance confirmed, highly likely to reduce herbicide performance), RR (resistance confirmed, probably reducing herbicide performance), R? (early indications that resistance might be developing, possibly reducing herbicide performance) or S (susceptible). It is used by all 13 organisations and companies undertaking resistance test in the UK, and has been used with both pot and Petri-dish assays with black-grass, rye-grass and wild-oats. It is described in Moss, S. R., *et al*. (1999). The occurrence of herbicide-resistant grass-weeds in the United Kingdom and a new system for designating resistance in screening assays. *Proceedings 1999 Brighton Conference - Weeds,* 179-184. See also references below.

**References and further information.**

Hull, R. & Moss, S. R. (2007). The 'Rothamsted Rapid Resistance Test' for ALS inhibiting herbicides: black-grass *(Alopecurus myosuroides)*. *Rothamsted technical publication*, 7pp. Rothamsted Research, Harpenden, UK. (this document).

Moss, S.R. (1999). The 'Rothamsted Rapid Resistance Test' for detecting herbicide-resistance in black-grass, wild-oats & Italian rye-grass. *Rothamsted technical publication*, 16pp.

Moss, S. R. (2007). The 'R' system for interpreting results from resistance screening assays in the UK. *Rothamsted technical publication*, 2pp.

The above three leaflets are available on request from the authors:

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Susceptible standard population
left dish = untreated, right dish = 0.1 ppm mesosulfuron+iodosulfuron

ALS target site resistant standard population
left dish = untreated, right dish = 0.1 ppm mesosulfuron+iodosulfuron