Crop Protection 69 (2015) 83–89

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Resistance profile of herbicide-resistant *Alopecurus myosuroides* (black-grass) populations in Denmark

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ARTICLE INFO

Article history:
Received 30 September 2014
Received in revised form
22 December 2014
Accepted 25 December 2014
Available online 3 January 2015

Keywords:
Black-grass
Herbicide resistance
ACCase inhibitor herbicides
Resistance pattern
Non-target-site resistance

ABSTRACT

Alopecurus myosuroides Huds is one of the most important grass-weeds in North-western Europe and is also the most important herbicide-resistant weed species in European agricultural systems. Fifty-three Danish *A. myosuroides* populations, previously confirmed to be fenoxaprop-P resistant, were evaluated for five and two known mutation points within the ACCase and ALS genes, respectively. The resistance pattern of 28 out of the 53 populations was investigated to four herbicides using a seed bioassay technique. A whole plant dose response experiment was conducted on seven populations in 2012 and 2013 to evaluate the accuracy of the seed bioassay results. Two resistant populations from the UK and a susceptible population from Denmark were included as reference populations in all experiments. Of the 53 populations, nine (17%) populations were ACCase target site resistant (TSR), all with a mutation at the Ile-1781 position. No mutations conferring TSR to ALS inhibitors were detected in the 53 populations. The seed bioassay results showed that all populations had varying degrees of resistance to fenoxaprop-P. In contrast, all populations were susceptible to cycloxydim suggesting that non-target site resistance (NTSR) was present in all populations including the ones where TSR were found. The seed bioassay was not a suitable method for detecting resistance to the residual herbicides pendimethalin and prosulfocarb. The whole plant dose response experiment results confirmed the results of the seed bioassay for fenoxaprop-P and cycloxydim. Three and two out of seven populations were also resistant to flupyrsulfuron and pendimethalin, respectively, while all resistant populations were susceptible to mesosulfuron + iodosulfuron. The widespread occurrence of particularly NTSR is a severe challenge to the effective management of *A. myosuroides*. In Denmark this challenge is even more prominent due to few modes of action being available for *A. myosuroides* control mainly due to national regulation on groundwater protection.

1. Introduction

Alopecurus myosuroides Huds (black-grass) is a major grass-weed in winter cereal crops in North-western European agricultural systems especially in the UK, France and Germany (Chavvel et al., 2002). It is a highly competitive weed, which can cause substantial yield reduction in winter wheat crops (Vizantinopoulos and Katranis, 1998). Vizantinopoulos and Katranis (1998) estimated the economic threshold level of *A. myosuroides* to be between 100 and 125 plants/m^2 in Greece but in England it was found to be only 12 plants/m^2 (Moss, 2013). *A. myosuroides* plants can produce many seeds (Lutman et al., 2013), thus a high level of control is required to prevent *A. myosuroides* populations from increasing (Lutman et al., 2013). More frequent cropping of winter crops, minimum tillage and early drilling (Colbach and Dürr, 2003), in addition to the rapid development of herbicide resistance (Heap, 2014), have caused an increase in *A. myosuroides* infestations.

A. myosuroides is the number one herbicide resistant weed in Europe (Lutman et al., 2013; Moss et al., 2007) having evolved resistance to six different modes of action and now ranked among one of the fifteen most important herbicide resistant weeds worldwide (Heap, 2014). Both target-site-based resistance (TSR) and non-target-site-based resistance (NTSR) have been reported in *A. myosuroides* populations (Délye, 2005).

ACCase-resistant *A. myosuroides* populations were found in the

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http://dx.doi.org/10.1016/j.cropro.2014.12.016
0261-2194/

UK in 1982 only five years after the first ACCase-inhibitor herbicide was introduced to the market. In Denmark, the first case of *A. myosuroides* resistance to ACCase inhibitors was reported in winter wheat in 2001 (Mathiassen, 2014). Since then many cases of resistance have been found in Denmark. The number of confirmed cases is at present 53 based on the testing of seed samples collected from fields with unsatisfactory control but the actual number of resistance cases is assumed to be higher (Mathiassen, 2014).

In France and the UK detailed studies have been conducted on resistant *A. myosuroides* populations. Délye et al. (2007) found that the majority (56%) of the French ACCase-resistant *A. myosuroides* populations were TSR. In contrast, NTSR seems to be dominating within populations in the UK (around 60%) and Germany (Drobny et al., 2006; Moss et al., 2007). Mapping the resistance pattern and mechanism of resistance among evolved herbicide resistant populations is important for developing sustainable herbicide resistance management programs (Beckie and Tardif, 2012).

The aims of this study were: a) to determine the molecular basis of resistance in the 53 resistant populations, b) to evaluate resistance pattern of Danish *A. myosuroides* populations collected between 2001 and 2012 in Petri-dish bioassay and c) to validate the results of the fast and cheap Petri-dish bioassay against the more time consuming and expensive whole plant bioassay for selected populations.

2. Materials and methods

2.1. Plant materials

Between 2001 and 2012, *A. myosuroides* seed samples were collected from 87 fields in Denmark where unsatisfactory weed control was reported. In preliminary whole plant tests 53 out of 87 were identified as being resistant to fenoxaprop-P with the first cases of resistance dating back to 2001 (Mathiassen, 2014).

In the present investigation all of these 53 populations were used for genotyping, 28 populations were included in Petri-dish assays and 7 populations were used in a whole plant dose response experiment. Two susceptible populations from Denmark (ID 85) and the UK (Roth99) respectively, and two resistant populations from the UK (Peldon and Notts) were included as standard reference populations. Peldon population exhibits a high level of NTSR giving multiple resistance patterns to chlorotoluron, diclofop, fenoxaprop-P, fluazifop-P, tralkoxydim, and pendimethalin (Hall et al., 1997; James et al., 1995). Recently also a low frequency of TSR to ALS herbicide mesosulfuron + iodosulfuron (mutation at position Pro-197) was detected in the Peldon population (Marshall and Moss, 2008). Notts is an ACCase TSR biotype with a mutation at the Ile-1781 position resulting in resistance to fenoxaprop-P, sethoxydim, diclofop and cycloxydim (Moss et al., 2003).

2.2. Molecular characterization of mutant ACCase and ALS alleles

In 2013, seeds of the 53 populations were sown in 2 L pots in a potting mixture consisting of soil, sand and peat (2:1:1 w/w). At the 2–3 leaf stage the pots containing 5–10 plants were sprayed with the recommended rate of 69 g a.i. ha $^{-1}$ fenoxaprop-P (Primera Super, 69 g a.i. L^{-1} fenoxaprop-P as the ethyl ester) in mixture with 0.2% of a non-ionic surfactant. Leaf material from one surviving plant was sent to a commercial company (IDENTXX, Stuttgart, Germany) for genotyping. The samples were analysed for seven point mutations identified in *A. myosuroides* ACCase gene (Ile-1781-Leu (two mutations), Trp-2027-Cys (two mutations), Ile-2041-Asn, Asp-2078-Gly and Gly-2096-Ala) (Délye et al., 2007) and 12 point mutations in *A. myosuroides* ALS gene at position Pro-197 with 10 point mutations and position Trp-574 with two known mutations (Tranel et al., 2014). Briefly, genomic DNA was extracted from resistant plants using a customized kit (Chemagic Plant400 Kit). PCR was performed using the specific primers of ALS and ACCase genes. PCR products of respective genes were amplified using initial denaturation for 5 min at 94 °C, followed by 35 cycles consisting of 94 °C for 40 s, 62 °C for 35 s, 72 °C for 30 s and 72 °C for 5 min for final extension. The PCR product was purified and analysed for single nucleotide polymorphism (SNP) using pyrosequencing (Qiagen, PyroMark Q24) according to manufacturer instructions.

2.3. Petri-dish germination assay

For the Petri-dish germination assay 28 of the 53 populations were selected. The populations were selected on basis of the year of sampling and seed availability. The resistance pattern to four herbicides (Table 1) was examined using the Rothamsted Rapid Resistance Test (Moss et al., 1999). Fifty seeds of each population were placed in 9-cm Petri-dishes containing three cellulose filter papers (Whatman No. 1) covered by one glass-fibre filter paper (Whatman GF/A). The discriminating concentrations for individual herbicides were determined in a preliminary experiment. Seven ml of each herbicide solution prepared with 0.1% potassium nitrate were added to each Petri-dish. There were two replicate dishes per population. For each population two control dishes containing 7 ml potassium nitrate solution were included. The Petri-dishes were stacked in zipped polythene bags to avoid loss of water by evaporation and placed in an incubator running a 17 °C/14 h light and 10 °C/10 h dark phase diurnal cycle. The herbicide treatments and replicates were kept in separate bags to avoid any cross contamination by herbicide vapour.

An empty Petri-dish containing three layers filter paper was placed on top of each stack so that the top dish received the same amount of light as the rest of dishes. Every other day the dishes were moved around in the incubator to keep conditions as uniform as possible across all treatments. The number of germinated seeds with shoots over 1 cm in each dish was counted after 2 weeks. The percentage reduction in number of seeds with shoots >1 cm relative to control dishes was calculated for all of populations and treatments. In addition to number of seeds with shoots over 1 cm the results were also evaluated according to the “R” rating system (Moss et al., 2007). According to this method plants exposed to a single discriminating dose are classified to four categories (RRR, RR, R? and S) relative to the responses of the susceptible standard population. RRR represents high resistance, RR; moderate resistance, R?; marginal resistance and S represents susceptible plants.

2.4. Whole plant dose response experiment

Seven out of the 28 populations included in the Petri-dish experiment were tested in dose response pot experiments in a glasshouse to evaluate their responses to six herbicides (Table 1). The populations were chosen to represent different resistance patterns from responses in the Petri-dish assays.

The experiments with soil applied herbicides (prosulfocarb and pendimethalin) was carried out in 1 L pots filled with a sandy loam field soil (69.3% sand, 13.9% silt, 14% clay, and 2.81% organic matter) collected in a field at the Dept. of Agroecology in Flakkebjerg. Fifteen seeds were sown in each pot and the pots were sub-irrigated with 250 ml water and moved to a glasshouse at 17/10 °C and a 16 h/8 h day/night cycle with supplemental light (180 μ mol m^{-2} s^{-1}). Herbicides were applied at six doses 24 h after sowing and 30 ml of water was applied to the soil surface to ensure an equal distribution of the herbicide into the top soil layer maximizing herbicide activity.

The experiments with the foliar applied herbicides (fenoxaprop-