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RESISTANCE OF WILD OAT (*Avena fatua*) POPULATIONS TO ACCASE-INHIBITING HERBICIDES AND MOLECULAR BASIS OF RESISTANCE

A. H. Muhammad Saiful[1], Qin Yu, Mechelle J. Owen and Stephen B. Powles

ABSTRACT

ACCase-inhibiting herbicides [aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD)] have been used extensively since late 1970s to control wild oat. However, continued reliance on ACCase-inhibiting herbicides has resulted in widespread evolution of resistant wild oat populations. Dose-response studies on four resistant wild oat populations determined the level of resistance to APP herbicides diclofop-methyl and fenoxaprop-p-ethyl, the CHD herbicides clethodim and sethoxydim, and to the phenylpyrazoline (PPZ) herbicide pinoxaden. All four resistant populations exhibited high level diclofop-methyl and fenoxaprop-p-ethyl resistance, but varied in level of resistance to other ACCase-inhibiting herbicides, indicating either different resistance mutations or different resistance mechanisms in these populations. Mutations of ACCase gene are known to endow target-site resistance to ACCase-inhibiting herbicides in other grass species. Therefore, we sequenced the carboxyl-transferase (CT) domain of the plastidic ACCase gene from ACCase-inhibiting herbicides surviving individuals to identify any mutations endowing resistance. In most, but not all individuals, three known amino acid substitutions endowing resistance were identified in resistant populations: the Ile-1781-Leu; Asp-2078-Gly; and Cys-2088-Arg. Polymerase chain reaction (PCR)-based marker analysis further confirmed the mutations are associated with resistance in these populations. Some of the individuals in one population also contained multiple (double and triple) mutations. Evidently, these mutations in ACCase gene endow high level of resistance in diploid grass species such as rigid ryegrass (Lolium rigidum) and blackgrass (Alopecurus myosuroides Huds). Whether the same mutations are to endow similar level of resistance in hexaploid wild oat remains to be investigated.

Keywords: *Avena* spp., ACCase-inhibiting herbicides, dose response and cross resistance patterns, ACCase gene, amino acids substitution, (d)CAPS.

INTRODUCTION

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2), the key enzyme involved in the first step of fatty acid biosynthesis in plants, is the primary target of ACCase-inhibiting herbicides (Délye, 2005). Three

[1] Australian Herbicide Resistance Initiative, School of Plant Biology, Institute of Agriculture, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

chemically distinct classes of herbicides; the aryloxyphenoxypropionates (APP), cyclohexanediones (CHD), and the recently developed phenylpyrazoline (PPZ) group with a single herbicide, pinoxaden (Hofer *et al*., 2006) are all herbicides that inhibit ACCase activity. Since the commercialization of the ACCase-inhibiting herbicides in the late 1970s, they have been extensively used to control many grass weed species. As a result of the great dependence on the ACCase-inhibiting herbicides, many grass weed species have evolved resistance to these herbicides. Currently, resistance to a single or cross-resistance to many ACCase-inhibiting herbicides has been documented in 40 grass species in 31 countries (Heap, 2011), with most ACCase resistance cases in ryegrass (*Lolium* spp.), blackgrass (*Alopecurus myosuroides* Huds) and wild oat (*Avena* spp.). Wild oat is one of the world's major grass weed species in the temperate cropping areas. Wild oat ranks only second to ryegrass as the most important herbicide-resistant weeds species globally (Heap, 2011). Evolved wild oat resistance to ACCase-inhibiting herbicides was first reported in Western Australia in 1990, in a wheat field (Piper, 1990). Since then, evolved resistant wild oat populations (mostly *Avena fatua* L. and *Avena sterilis* L.) have now become widespread in many countries including Australia (Heap, 2011).

Target-site ACCase gene mutations and non target-site enhanced metabolism have been established in resistant wild oat populations. For the ACCase gene mutations, eight distinct resistance endowing amino acids substitution have been implicated in several grass species: the Ile-1781-Leu substitution; the Trp-1999-Cys substitution; the Trp-2027-Cys substitution; the Ile-2041-Asn/Val; the Gly-2096-Ala substitution, the Asp-2078-Gly substitution; and the Cys-2088-Arg substitution (reviewed by Délye, 2005; Powles and Yu, 2010). Five of them (1781, 1999, 2027, 2041 and 2078) have been reported in ACCase herbicide resistant wild oat populations (Christoffers *et al*., 2002; Liu *et al*., 2007). A random survey across the very large Western Australia grain belt revealed that many wild oat populations have evolved resistance to ACCase herbicides (Owen and Powles, 2009). Four populations with resistance to several ACCase-inhibiting herbicides were selected for further investigation in this study. The objective of the research is therefore to quantify and compare the whole-plant resistance levels and cross-resistance patterns to ACCase-inhibiting herbicides, and to identify the molecular and biochemical basis of ACCase resistance in these populations.

MATERIALS AND METHODS

Whole-Plant Dose Response

All experiments were conducted during 2009 growing season (May-September) at University of Western Australia. Four resistant

wild oat (*Avena fatua* L.) populations, namely R1, R2, R3 and R4, plus a known susceptible population were used in this study. Plants were grown in pots outdoors under natural sunlight, watered and fertilized regularly. All seedlings were sprayed with selected ACCase-inhibiting herbicides at the 3-4-leaf stage.

Commercial ACCase-inhibiting herbicide formulations were used in all dose-response studies. ACCase-inhibiting herbicides: diclofop-methyl (Hoegrass®), fenoxaprop-p-ethyl (Wildcat®), clethodim (Select®), sethoxydim (Sertin®) and pinoxaden (Axial®) plus an appropriate adjuvant were applied using a custom-built, dual nozzle (TeeJet® XR11001) cabinet sprayer. All experiments were arranged in a randomized complete block design with three replications per herbicide per treatment. The herbicide rate required for 50% mortality (LD_{50}) and resistance index (R/S) were calculated.

Visual assessments of plant survival were made 21-day after herbicide treatment and the mean percentage of survival was calculated for each population. Shoot materials of individual plants surviving each ACCase-inhibiting herbicide at dosages that killed all the susceptible population plus untreated susceptible plants were sampled and stored at -80ºC for ACCase DNA sequencing and PCR-based marker analysis for ACCase resistance mutations.

DNA Extraction and ACCase Sequencing

All genomic DNA extraction and amplification procedures for both resistant and susceptible plants followed Yu *et al*. (2008) and Liu *et al*. (2007) under our specific modified PCR conditions. The PCR product was purified from agarose gel and sequenced using a commercial sequencing service.

PCR-based Marker Analysis

The published derived cleaved amplified polymorphic sequence (dCAPS) markers for 1781 (Kaundun and Windass, 2006) and 2078 (Kaundun, 2010) mutations were used in study with modified primers and PCR conditions, whereas a CAPS marker for 2088 mutation was developed. Restriction digestion and reaction procedures were carried out according to Yu *et al*. (2008).

RESULTS

Whole-Plant Dose Response to ACCase-inhibiting Herbicides

All four resistant populations were found to be highly resistant to diclofop-methyl. The diclofop-methyl LD_{50} value for the susceptible population was 454 g ai ha^{-1}, whereas LD_{50} values greater than 8800 g ai ha^{-1} (>20-fold) were recorded in all resistant populations (Table-1). Similar results were observed in response to fenoxaprop-p-ethyl. The fenoxaprop-p-ethyl LD_{50} values for resistant populations were 9 to >14-fold greater than susceptible population (Table-1).

For the CHD herbicide sethoxydim, the calculated LD_{50} values for resistant populations were 3 to 11 times higher than susceptible population (Table-1). Meanwhile, the level of resistance was much less for the herbicide clethodim. The LD_{50} values for resistant populations were 3 to 7 times higher than susceptible population (Table-1). For the herbicide pinoxaden, the LD_{50} of the resistant populations were 6 to 12 times higher than susceptible population (Table-1).

Table-1. The ACCase-inhibiting herbicides dose required for 50% mortality (LD_{50}) of the susceptible and resistant wild oat populations R1, R2, R3 and R4.

Herbicides	LD_{50} (g ha^{-1})				
	Susceptible	**R1**	**R2**	**R3**	**R4**
Diclofop-methyl	454	>8800	>8800	>8800	>8800
Fenoxaprop-p-ethyl	21	191	>308	>308	>308
Sethoxydim	104	272	1114	780	1121
Clethodim	4	9	12	11	24
Pinoxaden	3	19	20	12	40

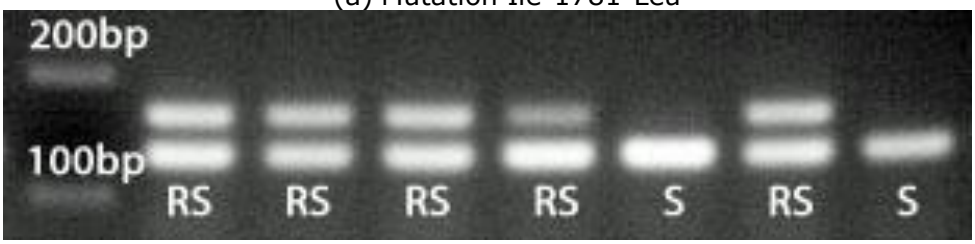
ACCase Sequencing Identified One to Three Mutations in a Single Resistant Plant

At least 38 individual plants surviving ACCase-inhibiting herbicides application from each resistant population were sequenced, and three known ACCase mutations were identified: Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg in all four resistant populations. While majority of the populations contain only a single mutation, interestingly, multiple (double and triple) mutations were identified in some individuals in population R2. It is also noted that while majority of the plants in populations R2 and R3 have ACCase mutations, 92% of resistant plants in population R1 and 27% in population R4 did not contain any of the known mutations.

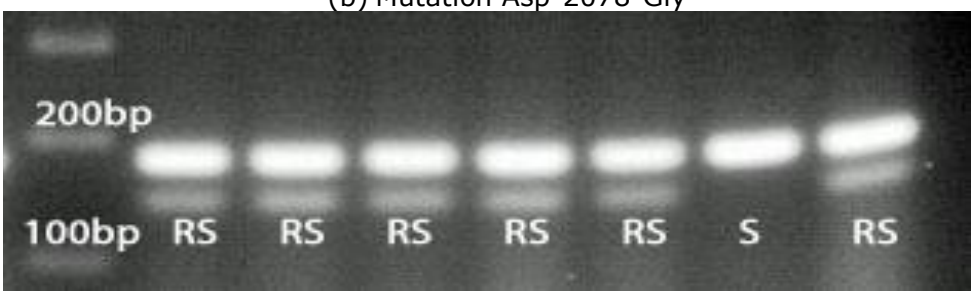
PCR-based Marker Analysis Revealed Fixed Heterozygosity for the Mutations in Individual Resistant Plants

ACCase sequencing revealed heterozygosity for each of the three mutations identified as double peaks (both mutant and wild type nucleotides) at the same position in the chromatograms. To confirm this nucleotide heterozygosity, three (d)CAPS markers were used to genotype sequenced individuals. Consistent with sequencing results, only heterozygous resistant (RS) or homozygous susceptible (S) but no homozygous resistant (R) genotypes were detected in a total of 157 samples analysed (Fig. 1).

(a) Mutation Ile-1781-Leu



(b) Mutation Asp-2078-Gly



(c) Mutation Cys 2088-Arg

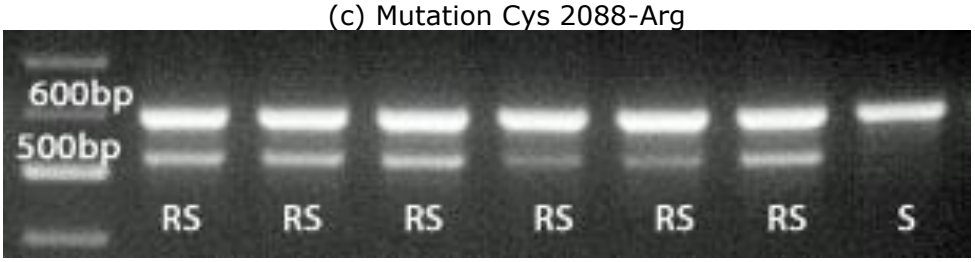


Figure 1. Genomic PCR based marker analysis of the ACCase mutations shows only homozygous susceptible (S) and heterozygous resistant (RS) genotypes for the: (a) 1781, (b) 2078 and (c) 2088 mutations detected in the ACCase-inhibiting herbicides surviving plants.

DISCUSSION

Results confirmed that cross-resistance to ACCase-inhibiting herbicides in wild oat populations in Western Australian grain belt is evident. All four resistant populations were found to exhibit high level of resistance to diclofop-methyl and fenoxaprop-p-ethyl, moderate level to sethoxydim and pinoxaden, and low level to clethodim. Differing patterns of resistance to ACCase-inhibiting herbicides in other wild oat populations have also been reported (Bourgeois *et al*., 1997; Uludag *et al*., 2007). The variations in resistance levels and resistance patterns exhibited by these populations were due to different

mutations or different resistance mechanisms (Maneechote *et al*., 1997).

In this study, the Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg mutations of ACCase gene were identified in four wild oat populations. One of the important findings of the current study was the identification of multiple ACCase mutations in individual plants. Thus, the role of target-site ACCase gene mutations in polyploid wild oat species truly needs further investigation. In addition to the ACCase target site mutations, many of the resistant individuals in populations R1 and R4 were found to have no known ACCase mutations in CT domain. Hence, it is likely that a single population can have both target-site and non target-site resistance mechanisms. Thus, further work is underway to confirm the non-target site resistance mechanisms in these populations.

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