Multiple Resistance to Acetyl Coenzyme A Carboxylase and Acetolactate Synthase Inhibiting Herbicides in Tunisian Ryegrass Populations (*Lolium rigidum*)

Haifa Hajri¹, Yosra Menchari² and Abdelwahed Ghrobel¹

1. Center of Biotechnology of Borj Cedria, University of Tunis El Manar, BP 901 Hammam-Lif 2050, Tunisia

2. Higher Institute of Biotechnology of Béja, University of Jendouba, Avenue Habib Bourguiba, BP 382, Béja 9000, Tunisia

Abstract: The good understanding of the mechanisms of resistance to herbicides in weeds is a necessity to implement sustainable weed management strategies. Here, a study was conducted to characterize the molecular bases of resistance to acetyl coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS) inhibiting herbicides in *Lolium rigidum* populations from Tunisia. Nine *Lolium rigidum* (ryegrass) populations collected in wheat fields from Northern Tunisia were investigated for their resistance to two ACCase-inhibiting herbicides and an ALS-inhibiting herbicide. All populations were tested in the greenhouse in pots using the commercial dose to determine resistance status. Survival plants were also tested for the presence of two ACCase (L1781 and N2041) and two ALS (P197 and W574) mutant resistant alleles using molecular markers. Resistance to ACCase-inhibiting herbicides was found in all tested populations. Comparison of the results from herbicide sensitivity bioassays with genotyping indicated that more than 80% of the plants resistant to ACC-inhibiting herbicides would be resistant via increased herbicide metabolism. However, ALS-inhibiting herbicides are still more or less controlling ACCase resistant populations, so indicating that the selection process of resistance is ongoing. Target-site resistance appears to be the major mechanism for these early cases of ALS inhibitor resistance. This study reported the first case of resistance to ALS-inhibiting herbicides in ryegrass in Tunisia, and investigated the molecular bases of this resistance. It establishes the clear importance of non target-site resistance to ACCase- and/or ALS-inhibiting herbicides.

Key words: ACCase, ALS, cross resistance, *Lolium*, mutant resistant alleles, metabolism.

1. Introduction

In most agricultural areas worldwide, herbicides have become the major tool of weed control. The intense selection pressure exerted by herbicides has resulted in the development of herbicide resistance in many weed species as the consequence of the adaptive evolution of weed populations [1, 2].

Lolium rigidum (ryegrass) is one of the most troublesome herbicide-resistant weeds. It has evolved resistance to 11 herbicide modes of action [3]. Among herbicides having been selected for resistance in ryegrasses are acetolactate synthase (ALS, EC 2.2.1.6), also known as acetohydroxyacid-synthase (AHAS) inhibitors, and acetyl-CoA carboxylase (ACCase, EC

6.4.1.2) inhibitors [4-9].

ALS is the first common enzyme in the biosynthesis of leucine, isoleucine and valine [10]. ALS inhibitors are among the most used herbicides worldwide [11, 12]. AHAS is the common target site of five AHAS-inhibiting herbicide chemistries, namely sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine, pyrimidinyl-thiobenzoates and sulphonyl-aminocarbonyltriazolinone.

Resistant weeds can survive from herbicide application via a variety of mechanisms that can be divided into two categories: target-site resistance (TSR) mechanisms, including increased expression of the target protein or structural changes to the herbicide binding site, and non-target-site resistance (NTSR) mechanisms, including any other mechanisms not belonging to TSR [13]. While the genetic basis of

Corresponding author: Yosra Menchari, assistant professor, research field: plant biotechnology.

NTSR resistance is still obscure, TSR resistance is much better understood.

Resistance to ALS inhibitors can be due to mutations in the ALS gene that decreases the affinity of the ALS enzyme for herbicides, or to other mutations causing enhanced degradation of inhibitor molecules by plant metabolism [10]. A total of 22 resistance-endowing gene mutations at seven conserved amino acid residues in the ALS gene have so far been identified in field-evolved resistant weed biotypes [10, 11, 14].

To date, mutations at Pro-197 (especially the Pro-197-Ser) and at Trp-574 are the most commonly reported [9, 14]. These two mutations confer resistance to SU and IMI families.

ACCase is a key enzyme in lipid biosynthesis that catalyzes the formation of malonyl-CoA from the carboxylation of acetyl-CoA. ACCase herbicides, introduced since 1978, have become widely used for grass weed control in world agriculture. There are many ACCase herbicides across the aryloxyphenoxypropionate (APP), cyclohexanedione (CHD) and phenylpyrazoline (PPZ) chemical groups. TSR results from a single amino acid change in the ACCase enzyme. To date, mutations at seven ACCase codons have been reported at positions 1781, 1999, 2027, 2041, 2078, 2088 and 2096 (*Alopecurus myosuroides* equivalent) [10, 15]. The cross resistance patterns between currently commercial ACCase herbicides have been established in some weed species for some of these resistance mutations [10, 15].

The first case of ryegrass resistance to an ACCase herbicide was reported in 1982 in a wheat field in Australia [16]. Over the last 30 years, many more ryegrass populations have evolved resistance to ACCase-inhibiting herbicides across the five continents [2]. In Tunisia, the first case of herbicide resistance was reported in 1996. It concerned *Lolium rigidum* (ryegrass) populations collected in wheat fields from Northern Tunisia and found to be resistant to ACCase-inhibiting herbicides [17]. These

herbicides have been broadly used to control grass weeds since the end of the 80s. As an alternative solution, farmers have come to rely on ALS-inhibiting herbicides and control failures have been observed, which suppose the development of ALS resistant *Lolium* populations.

The objective of this study was to investigate the molecular bases of resistance to ACCase- and ALS-inhibiting herbicides in nine Tunisian *Lolium* populations.

2. Materials and Methods

2.1 *Lolium* Populations

Nine populations of ryegrass (*Lolium rigidum*) were collected in 2009 from different wheat fields in Northern Tunisia (the governorate of Bizerte), where the first case of resistance to ACCase-inhibiting herbicides was reported in 1996 [17]. Seeds were randomly sampled from a large number of surviving plants across the entire field.

In the absence of a susceptible population, an Italian population displaying 20% of resistance to clodinafop and susceptible to ALS-inhibiting herbicides was used as a control. Resistance level was previously screened using a quick test experiment with Petri dishes containing agar and herbicide solution. No specific permissions were required for the locations where ryegrass seeds were collected.

2.2 Whole-Plant Herbicide Sensitivity Assessment

Seeds from each population were first placed in Petri-dishes containing a KNO₃ solution (20 mM) to increase germination rate, and incubated in a culture chamber at 18 °C. Around 15 germinating seeds were sown at 2 cm deep in individual pots of 15 cm diameter containing a 1:1 ratio of sand and peat, and then irrigated as necessary. Pots were maintained in a controlled greenhouse set at 25 °C day for 16 h and 18 °C night for 8 h. Seedlings at the two- to three-leaf stage were separately, and treated with the commercial's recommended application doses of the

commercial formulations of two ACCase-inhibiting herbicides, i.e., clodinafop-propargyl + cloquintocet-2-mexyl (Topik 100, 0.7 L/ha, Syngenta) and pinoxaden + clodinafop-propargyl (Traxos 45 EC, 1.2 L/ha, Syngenta), and an ALS-inhibiting herbicide, i.e., iodosulfuron + mesosulfuron (Amilcar, 30 g/ha, Bayer) using a moving nozzle sprayer at a rate of 200 L/ha. For each population investigated including the reference one, 15 additional plants were sprayed with water (untreated control). The pots were placed in a completely randomized design and three replicates were used for each treatment. Twenty-one days after treatment, the number of surviving plants in each pot was visually assessed for the ACCase inhibitors. As ALS-inhibiting herbicides causes stunt in grass weeds, the aboveground biomass was harvested, dried for 3 d at 65 °C and weighted.

2.3 Genotyping of ACCase and ALS Mutations

A 1 cm section was cut from the first leaf of surviving seedlings in each population. Each leaf section was placed with a glass bead (2 mm diameter) into a 0.2 mL microcentrifuge tube containing 75 µL of extraction buffer (100 mM Tris-HCl, pH = 9.5, 1 M KCl, 10 mM ethylenediaminetetraacetic acid (EDTA)).

Leaf fragments were disrupted using a bead mill TissueLyser (Qiagen, Courtaboeuf, France). Tubes were placed in a water bath at 95 °C for 6 min, transferred onto ice for 5 min and vortexed for 15 s. DNA extracts were diluted 50-fold and kept at -20 °C before genotyping.

Published markers have been used as described in Refs. [4, 18, 19] to accurately detect the L1781 and N2041 ACCase mutations and P197 and W574 ALS mutations in Tunisian ryegrass populations (Table 1). All PCR amplifications were performed in 20 µL reaction mixes [18]. Primers were used at 0.2 µM each (Table 1). Cycling programs consisted of 95 °C for 5 min, followed by 37 cycles of 95 °C 5 s, annealing temperature for 10 s and 72 °C for 30 s each. For cleaved amplified polymorphic sequence (CAPS) and derived cleaved amplified polymorphic sequence (dCAPS) techniques, digestions were performed for 3 h at 37 °C, except for ApaI enzyme (30 °C). The 10 µL digestion mixes contained 4 µL of the PCR mixes, 5 U enzyme (Fermentas, Vilnius, Lithuania), 0.5 µL 10× provided enzyme buffer and 4 µL water. Patterns were visualized by electrophoresis on 3% (w/v) agarose gels for CAPS and dCAPS and 1.3% agarose gels for allele-specific PCR, run in 0.5 TBE buffer.

Table 1 Primers and markers used for detection of ACCase and ALS mutant resistant alleles.

Gene	Mutation	Primers**	Sequence 5'—3'	Annealing temperature	Restriction enzyme	Technique
ACCase	Leu-1781*	VRDIC+	GGACTAGGTGTGGAGAACC	63 °C	-	Gene-specific PCR [18]
		VRDITR	CAATAGCAGCACTTCCATGTAA			
		ACVRG1	AATGGGTCGTGGGGCACTCCTATAATTCC			
		ACVRG1R	GCTGAGCCTCAATATATTAGAAACAAC			
ACCase	Asn-2041	ACCF1	CACAGACCATGATGCAGCTC	62 °C	EcoRI	CAPS [19]
		ACCR1	CTCCCTGGAGTTGTGCTTTC			
	Gly-2078	ACCF1 EcoRV2078r	CACAGACCATGATGCAGCTC GCACTCAATGCGATCTGGATTATCTTGATA	62 °C	EcoRV	dCAPS [19]
ALS	Pro-197	RG197F	TCCTCGACTCCATCCCCATGGTGGCCATCACG	60 °C	Apa I	dCAPS [4]
			GGGCAGGGC			
		RG197R	GCGGCCAGAGGAGGCGAGGAAGAAGG			
	Trp-574	RG574F	GGTGATGATACTGAACAATCAACATCTTGGA	65 °C	BstXI	dCAPS [4]
			ATGCCAGTGCAG			
		ALV7R	ATACACCAGCATCATGCTGATCAGG			
	Ser-653	RG653F	ATCATCGTCCCTCACCAGGAGCATGTGCTGC	60 °C	Bpu10I	dCAPS [4]
			CTATGATCCTT			
		ALV7R	ATACACCAGCATCATGCTGATCAGG			

*Amino acid positions correspond to the full length in *Alopecurus myosuroides*.

**Primer name ending with F (forward primer) and R (reverse primer).

2.4 Statistical Analysis

Data were subjected to ANOVA to test significant differences between the resistant and the sensitive populations in response to each herbicide treatment using the PROC GLM statement in SAS (version 9.3, SAS Institute, Cary, NC).

3. Results

3.1 Resistance Confirmation Test

Screening of *Lolium* populations collected from wheat fields showed that there were very high frequencies of populations that are resistant to the ACCase-inhibiting herbicides. For clodinafop, a significant difference between the nine populations collected and the reference population used as a control was observed. The percentage of survivors varies between 31% and 94% (Fig. 1). The population used as a reference has a rate of 17% of surviving plants, not significantly different from the control level of 20% ($\text{K}\chi^2 = 3.84$, $P = 3.3\text{E}^{-8}$). For the test with clodinafop + pinoxaden, only one population was sensitive. The other eight populations are displaying high frequencies of surviving plants varying between 38% and 100% (Fig. 1).

For the ALS-inhibiting herbicide, significant biomass reductions of 47% and 54% were observed in populations P4 and P8, respectively, compared to the

Italian standard population (Fig. 2).

3.2 Molecular Bases of Resistance to ACCase- and ALS-Inhibiting Herbicides

Within the carboxyl-transferase (CT) domain of the plastidic ACCase gene, published markers were used to detect L1781 and N2041 mutations. In previous studies, these mutations are the most frequently observed in different grass weed species [19, 20].

The L1781 mutation was detected in eight out of the nine *Lolium* populations studied. Among the 124 surviving plants analyzed, 10 plants were homozygous mutant (8.1%), 29 were heterozygous mutant (23.4%) and 85 were wild type (68.5%) (Table 2).

For the N2041 mutation, a total of 71 plants were genotyped. Three plants were homozygous mutant (4.2%), 14 were heterozygous mutant (19.7%) and 54 were wild type (76.1%) (Table 2). The mutant resistant alleles were detected in five populations (P2, P3, P4, P6 and P8).

A total of 260 plants from the nine ryegrass populations were genotyped to detect the presence of P197 and W574 mutations. For the P197 mutation, three plants were homozygous mutant (1.2%), 20 were heterozygous mutant (7.7%) and 237 were wild type (91.1%) (Table 2). It was detected in five populations and was the most prevalent in the population P4. For the W574 mutation, two plants were homozygous

Fig. 1 Sensitivity of the 10 ryegrass populations to clodinafop (a) and to clodinafop + pinoxaden (b) estimated by the percentage of survival plants.

Fig. 2 Sensitivity of the 10 ryegrass populations to iodosulfuron + mesosulfuron estimated by the percentage of reduction of biomass related to the untreated control.

Table 2 Molecular analysis for plants in the *Lolium* populations investigated for ACCase and ALS resistant alleles.

Variable	ACCase alleles								ALS alleles							
	L1781				N2041				P197				W574			
	RR	RS	SS	Total	RR	RS	SS	Total	RR	RS	SS	Total	RR	RS	SS	Total
No. of plants	10	29	85	124	3	14	54	71	3	20	237	260	2	12	246	260
Frequency (%)	8.1	23.4	68.5	100.0	4.2	19.7	76.1	100.0	1.2	7.7	91.2	100.0	0.8	4.6	94.6	100.0

RR for homozygous mutant plants, RS for heterozygote and SS for wild-type.

mutant (0.8%), 12 were heterozygous mutant (4.6%) and 246 were wild type (94.6%) (Table 2). It was detected in five populations.

Different mutant alleles can be present within the same *Lolium* population. In this study, seven populations (P1, P2, P3, P4, P7, P8 and P9) contain at least one ACCase and one ALS mutant resistant alleles studied. In the P2 and P8 populations, the four alleles studied were detected.

4. Discussion

This is the first study to investigate the molecular bases of resistance to ACCase- and ALS-inhibiting herbicides in Tunisian *Lolium* populations, and the first to report resistance to ALS-inhibiting herbicides. Assessment of the sensitivity of nine ryegrass

populations to two ACCase-inhibiting herbicides revealed that resistance is very developed. In Tunisia, these herbicides were broadly used for the control of grass weeds in cereal crops, which explain the high levels of resistance observed. It has been observed that the average number of resistant plants was weakly correlated with the number of applications of ACCase inhibitors [21, 22].

High levels of resistance have been observed with clodinafop + pinoxaden. The PPZ herbicide pinoxaden has been used in Tunisian wheat field since 2009 (the year when the author collected the populations), which indicates that resistant individuals to this herbicide were already present in the field and selected by other ACCase-inhibiting herbicides. A study conducted on *Alopecurus myosuroides* Huds. in France, where

resistance to pinoxaden have been observed, showed that the average frequency of resistant plants computed for pinoxaden also increased with the number of clodinafop applications [22]. Similar results were also obtained on Italian *Lolium* populations [19]. This is consistent with the genotyping results in this study. In fact, in *Lolium rigidum*, the L1781 resistant alleles confer cross resistance to all APP, CHD and PPZ herbicides [10]. This mutation was detected in almost all populations studied.

The assessment of the sensitivity of the nine ryegrass populations showed that resistance to ALS-inhibiting is less developed than resistance to ACCase-inhibiting herbicides, and seems to be emerging. It has so far been detected in five countries [18], but this study represents the first report of this resistance in Tunisian *Lolium* populations.

Resistance to ACCase- and ALS-inhibiting herbicides was both observed within Tunisian *Lolium* populations. Biotypes that are resistant to herbicides from two or more mode-of-action families as conferred by more than one physiological mechanism are termed multiple herbicide resistant (MHR) [23]. This type of resistance makes weed management more challenging, because changing to a different mode of action may not control MHR biotypes. Besides, at least two different mutant alleles (ACCase and/or ALS alleles) were detected in the same population. This is to be expected in this highly genetically diverse, obligate cross-pollinated grass weed.

By computing the cross resistance patterns of L1781 and 2041 ACCase alleles, P197 and W574 ALS alleles and their respective frequencies in each population determined by genotyping, the expected proportions of plants to be resistant to clodinafop and iodosulfuron + mesosulfuron, respectively, were obtained. The comparison between these expected proportions with the observed ones obtained from pot bioassays revealed an excess of observed resistant plants to clodinafop in the nine *Lolium* populations. The ACCase alleles explain less than 20% of the

resistance observed in ryegrass populations. The other part can mainly be due to NTSR. However, the mutant ALS resistant alleles account for most of the resistance to sulfonylurea.

NTSR is now considered the predominant type of resistance to the first and second most important herbicide groups worldwide, i.e. ACCase and ALS inhibitors in grasses [13]. For ryegrass, it was shown that an increased activity of a P450 conferred cross-resistance to ALS-inhibiting herbicides and ACCase-inhibiting herbicides [24].

5. Conclusions

This study has confirmed the presence of high levels of resistance to ACCase-inhibiting herbicides and reported for the first time resistance to ALS inhibitors in ryegrass Tunisian populations. However, this resistance is partly due to TSR. NTSR seems to be very widespread. It is a very complex phenomenon that cross resistance patterns could be in some cases very unpredictable and involve a wide range of herbicides that have even not yet used, thus complicating weed management. As a result, in the context of the dearth of innovation and increases of resistance, a herbicide becoming ineffective because of resistance is increasingly difficult to replace, and each herbicide molecule available is very valuable and should therefore be used as long as possible with a maximum of efficiency.

Considering the likelihood of multiple and NTSR resistance mechanisms in the same population, the implementation of integrated weed management practices will increasingly need to complement chemical means.

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