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Cross-and Multiple Herbicide Resistant *Lolium rigidum* Guad. (Rigid Ryegrass) Biotypes in Iran

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ABSTRACT

Weed competition, especially from grass species, is estimated to cause 23% reduction in yield in the wheat fields of Iran. During the years 2013 to 2016, a study was conducted to evaluate the resistance to herbicides of 30 rigid ryegrass (*Lolium rigidum*) biotypes that had been collected from wheat fields of Khuzestan Province. The screening of these biotypes was conducted with clodinafop-propargyl in the greenhouse and revealed biotypes with a survival rate of greater than 20% in response to this herbicide. These biotypes were further studied for the evaluation of cross and multiple resistance. A total of 94 and 75% of the rigid ryegrass biotypes showed resistance to ACCase- and ALS-inhibitors, respectively. Approximately 69% of the rigid ryegrass biotypes included individuals with resistance to at least two herbicide mechanisms of action. This is the first report of cross and multiple resistance in rigid ryegrass biotypes from Iran. The leaves of the rigid ryegrass biotypes cross-resistance to ACCase-inhibitors were analyzed using CAPS and dCAPS markers to identify probable amino acid substitutions at 2,041, 2,088, 1,781, and 2,078 positions on the *ACCase* gene. In two and nine biotypes, mutations were observed in the 1,781 and 2,041 positions, respectively. These results indicated that there is a serious problem with herbicide resistance in rigid ryegrass, including cross and multiple resistance, and a need to implement long-term integrated management strategies.

Keywords: ACCase inhibitors, ALS inhibitors, CAPS markers, dCAPS markers, Mutation.

INTRODUCTION

Herbicide resistance is a ubiquitous challenge to herbicide sustainability and a looming threat to the control of weeds in crops (Mahmood *et al*., 2016). Herbicide Resistance (HR) can be defined as the acquired ability of a weed population to survive and reproduce after application of an herbicide that was previously known to control that population. In a plant, this resistance can be inherent or it can be induced by techniques such as genetic engineering or by a selection of plants created by tissue culture or mutagenesis (Vencill *et al*., 2012; Kaundun, 2014).

Rigid ryegrass is widely distributed throughout many countries (Loureiro *et al*., 2010; Rauch *et al*., 2010; Heap 2017), and is the most significant weed in Iranian crop production systems (Montazeri *et al*., 2005). Consequently, it is one of the species most targeted for control during grain production in most areas of the country. Herbicides are used to control this weed in crop production systems because of their high efficacy, ease of

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use, and cost effectiveness. Grass-selective Acetyl Coenzyme A Carboxylase (ACCase, EC 6.4.1.2) inhibitors in particular have been widely used (Busi *et al*., 2017).

Three chemically distinct classes of herbicides that are known to inhibit ACCase are the hydroxyphenoxyisopropionic acid (AOPP or ArylOxyPhenoxyPropionate), hydroxyoxocyclohexenecarbaldehyde oxime (CHD or CycloHexanoDione) and phenyloxopyrazolinyl formate (PPZ or PhenylPyraZoline) chemical families (Forouzesh *et al*., 2015). In grasses, plastids contain the eukaryotic form of ACCase and are sensitive to three chemical classes of herbicides known as graminicides. Most dicotyledonous plant species contain the prokaryotic form of the enzyme that is insensitive to graminicide (Vencill *et al*., 2012). It has been reported that over 80% of the activity of the ACCase enzyme is related to the plastid isoform, although the Poaceae family only has the homomeric form in both the plastids and cytosol (Konishi and Sasaki, 1994; Huerlimann and Heimann, 2013). Resistance to ACCase and AcetoLactate Synthase (ALS, EC 2.2.1.6; also referred to as AcetoHydroxyAcid Synthase: AHAS)-inhibiting was reported in many populations of major grass weeds, such as the wild oat (*Avena fatua* L., *A. sterilis* L.) and rigid ryegrass (Cruz-Hipolito *et al*., 2011; 2015; Travlos *et al*., 2011, 2013; Adamczewski *et al*., 2013; Rosenhauer *et al*., 2013; Heap, 2017).

Since the introduction of ACCase-inhibitors in the late 1970s, they have been used worldwide to selectively control grass weed species in winter cereals (Powles and Yu, 2010). In recent decades, there have been increasing numbers of reports on graminicide-resistant weeds. Heap (2017) has reported on graminicide resistance in 47 grass weed species around the world. In Iran, herbicides such as clodinafop-propargyl, diclofop-methyl, fenoxaprop-P-ethyl and pinoxaden have been commonly used post-emergence for many years to control grass weeds such as *Lolium* spp., *Avena* spp. and *Phalaris* spp. in wheat (Zand *et al*., 2010).

Two types of mechanisms are involved in resistance (Beckie and Tardif, 2012; Délye, 2013). TSR (Target-Site Resistance) mechanisms are related to conversion in a three-dimensional combination of the herbicide target protein that prevents herbicide action or by enhancing the action of the target protein. TSR is frequently reported in resistant weed species and is endowed with gene mutations in target enzymes such as ALS and ACCase (Yang *et al.*, 2016; Matzrafi *et al*, 2017). NTSR (Non-Target-Site Resistance) mechanisms are related to a decrease in the herbicide uptake and/or translocation or increased herbicide detoxification (Délye, 2013; Yuan *et al*, 2007). NTSR may cause weeds to evolve unpredictable resistance to herbicides with varied modes of action, even to herbicides that are not currently being marketed (Yu and Powles, 2014; Délye *et al.*, 2015). Compared to TSR, NTSR is less studied and little-known because of its complexity and variety.

Wheat is an important and strategic product of Iran, but its production has been reduced by grass weeds such as rigid ryegrass. The control of this weed in Iranian wheat fields has been mainly by the use of ACCase-inhibiting herbicides. The objectives of the current study were to: (1) Confirm the resistance of rigid ryegrass to AOPP herbicide; (2) Study cross-resistance patterns to ACCase- and ALS-inhibiting herbicides by means of dose-response experiments; (3) Study multiple resistance patterns in both ACCase-inhibiting and ALS-inhibiting herbicides such as clodinafop-propargyl and chlorsulfuron, respectively, and resistance to other herbicide groups; and (4) Identify possible *ACCase*-gene mutations that endow cross-resistance to ACCase-inhibiting herbicides in these biotypes by molecular methods.

MATERIALS AND METHODS

Collection of Plant Material

Mature spikes of rigid ryegrass plants (29 putative ACCase-inhibitor resistance

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biotypes) that had survived repeated ACCase-inhibiting herbicide application were collected in wheat fields of the mid-west region of southwestern Iran, in 2013. Spikes from a susceptible biotype (HF), used as the control, were collected from a region with no history of ACCase-inhibiting herbicide application (Figure 1). When rigid ryegrass occurred in large patches, approximately 20 spikes were collected from the patch. Sampling was completed when approximately 50-100 spikes had been collected. The spikes were placed in labeled paper bags and the coordinates of each field were recorded (by GPS). All the spikes from one field were pooled and designated as a single population. The samples were stored at room temperature (20-25°C) for six months over the summer to allow the seeds to completely dry and be released from dormancy. The seeds were then cleaned by hand and stored in paper bags at room temperature (Zand *et al.*, 2008).

Plant Growth and Resistance Evaluation

Screening Test

After seed dormancy was broken (treatment with gibberellic acid at a concentration of 10 ppm), 10 germinated seeds were sown at a depth of 1-2 cm in 500 mL plastic pots filled with a manure-loam-sand mixture (1:1:1 by volume) and the pots were watered as required until harvest. Shortly after emergence, the plants were thinned to a final density of seven seedlings per pot. Clodinafop-propargyl (Topik; Syngenta, 64 g ai ha^{-1}) belonging to the AOPP chemical classes were applied to

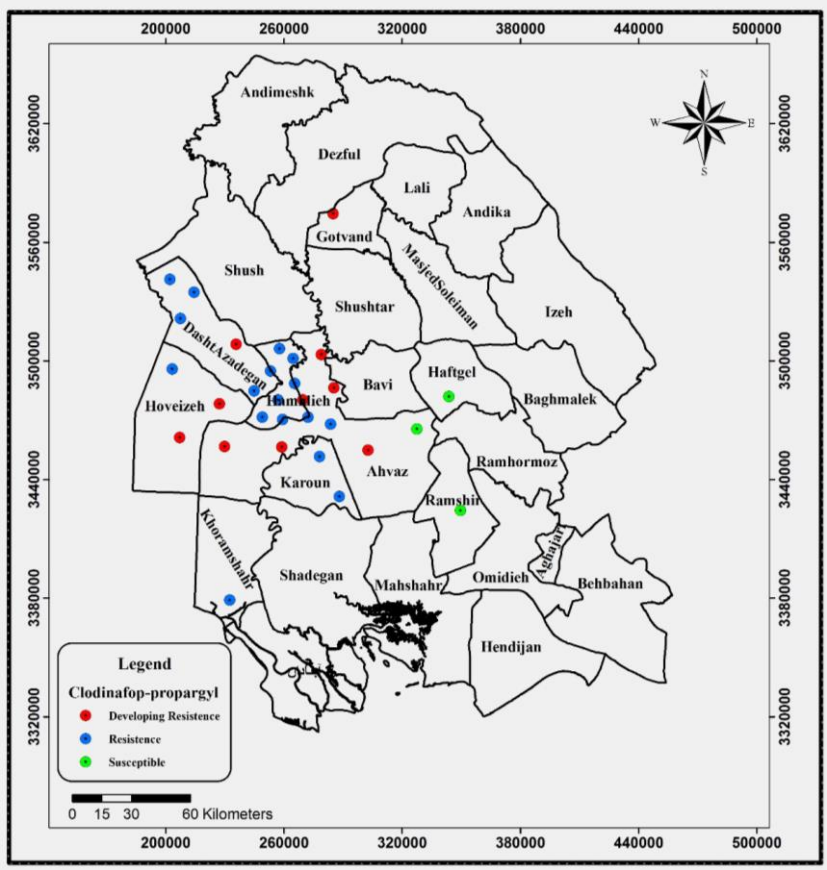

Figure 1. Map of Khuzestan province, showing the approximate locations where rigid ryegrass populations were collected.

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plants of the resistant and susceptible rigid ryegrass biotypes at the 2-3 leaf stage. The herbicide was applied with a laboratory sprayer (Marolex; 12 L) equipped with a single TeeJet flat-fan nozzle (8001) delivering 250 L ha^{-1} at 200 kPa. The experiment had a completely randomized design with four replications.

Differences between the seed biotypes were evaluated at 28 days after treatment as the percentage compared to the untreated control. Afterwards, the biotypes were classified according to the number of plants that survived each herbicide treatment. Susceptible biotypes were classified as those having 0% plant survival, biotypes developing resistance were those with 1 to 19% survival, and resistant biotypes were those having greater than 20% survival rate according to the classification proposed by Owen *et al*. (2007). Biotypes with survival rates above 20% compared to the control were further separated and selected for cross-resistance and multiple resistance evaluation.

Evaluation of Performance of Herbicides Groups by Biotype

In this experiment, 15 previously confirmed resistant biotypes and one biotype susceptible to clodinafop-propargyl ryegrass were treated with several herbicides. The experiment was conducted as a completely randomized design with four replications. Ten germinated seeds were sown at a depth of 1-2 cm in 500 mL plastic pots filled with manure-loam-sand mixture (1:1:1 in volume) and the pots were watered as required until harvest. Shortly after their emergence, the plants were thinned to a final density of seven seedlings per pot. The pots were fertilized as required and placed under conditions of 20-25°C/16 hours day, 10-15°C/8 hours night, and 65% relative humidity. The required light was provided by 900 µmol^{-2} s^{-1} photosynthetic photon flux density delivered by fluorescent and incandescent lights. The herbicides shown in Table 1 were applied to the plants of the resistant and susceptible rigid ryegrass

Table 1. Herbicides used in this study.

Chemical families	Mechanisms of action [d]	Active ingredient	Commercial product	Field rate (g ai ha^{-1})
AOPP [a]	ACCase	Clodinafop-propargyl	Topik®, Syngenta	64
		Clodinafop-propargyl+Oil	Topik®+Oil	64
CHD [b]	ACCase	Diclofop-methyl	Illoxan®, Syngenta	900
		Clethodim	Select®, Syngenta	120
		Sethoxydim	Vantage®, BASF	375
PPZ [c]	ACCase	Pinoxaden	Axial®, Syngenta	45
		Pinoxaden	Axial®, Syngenta	60
AOPP + PPZ	ACCase	Clodinafop-propargyl+ Pinoxaden	Traxos®, Syngenta	67.5
Sulfonylurea	ALS	Chlorsulfuron	*Megaton*®, Du Pont	15
		Mesosulfuron-methyl+ Iodosulfuron-methyl sodium	Atlantis®, Bayer	180
		Sulfosulfuron+Metsulfuron	Total®, Bayer	36
Phenylurea	PSII+CB	Isoproturon+Diflufenican	Panther®, Bayer	1375
Bipyridyl	PSI	Paraquat	Gramoxon®, Syngenta	600
Glycine	EPSPS	Glyphosate	Roundup®, Bayer	1640

[a] ArylOxyPhenoxyPropionate; [b] CycloHexanoDiones, and [c] PhenylPyraZolines. [d] Herbicides inhibitors of: ACCase= Acetyl CoA Carboxylase; ALS= AcetoLactate Synthase or acetohydroxy acid synthase; EPSPS= 5-EnolPyruvylShikimate-3-Phosphate Synthase; PSII= PhotoSystem II, and CB= Carotenoid Biosynthesis.

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biotypes at the 2-3 leaf stage, except those treated with isoproturon+diflufenican, which was applied pre-emergence (3 days after sowing seedlings). Herbicide treatments were made with a laboratory sprayer (Marolex; 12 L) equipped with a single TeeJet flat-fan nozzle (8001) delivering 250 L ha^{-1} at 200 kPa. The herbicide efficacies were represented as percentage of survival compared to the untreated plants (control) at 28 days after treatment (Owen *et al*., 2014).

Molecular Methods

DNA Extraction

DNA was extracted from the leaves (0.5 to 1.0 g) by using the CTAB method (Henderson and Hammond, 2013). The quality and quantity of the DNA were determined by spectrophotometer and agarose gel, respectively. In this study, the possible mutations in the *ACCase* gene were studied by two methods, CAPS (Cleaved Amplified Polymorphic Sequences) and dCAPS (derived Cleaved Amplified Polymorphic Sequences). The concentrations of material used in the PCR reaction S and the enzymatic digestion were similar (Yu *et al.*, 2007).

The PCR reactions for both methods were performed in a final volume of 25 µL (1.0 µL of DNA 50 ng μL^{-1}, 2.5 µL of PCR buffer 10X, 0.75 µL of $MgCI_2$ 50 mM, 0.5 µL of dNTP mix 20 mM, 1.0 µL of Primer F100 Pmol μL^{-1}, 1.0 µL of primer denaturation R100 Pmol μL^{-1}, 0.2 µL of Taq polymerase of 5 u μL^{-1}, and 18.8 µL of H_2O_2). Amplification was performed using an automated DNA thermal cycler (FlexCycler² Analytik Jena, Germany). The PCR was performed for 35 cycles of 94°C, 30 seconds of annealing at 60°C and 2 minutes of elongation at 68°C, and a final extension for 10 minutes at 72°C. In a study on the mutation of Isoleucine-2041-Asparagine, the expansion of the new sequence occurred at 72°C in 1 min instead of 30 seconds. The enzyme digestion reaction in an extremity volume of 30 µL was completed in 16 hours at 37°C. The volume of material needed for one enzyme reaction for each of the two tests included the PCR product (10 µL), 10X buffer R (2 µL), Enzyme (1 µL), and H_2O_2dd (17 µL).

CAPS Analysis

The primer pair ACCFI/ACCRI was used to amplify a 492-bp segment of ACCase followed by restriction with EcoRI (Yu *et al.*, 2007). Following observation of the digestion bands, homozygous- and heterozygous-resistant and homozygous susceptible plants were classified. The mutation of thymine to cysteine at codon 2088 replaced cysteine to arginine to create a restriction site for the Eco47ш enzyme. (Table 2-3).

dCAPS Analysis

Nsil 1781f/Nsil 1781r primers were used to amplify a 165-bp segment of ACCase followed by restriction with Nsi (Kaundun and Windass, 2006). A dCAPS marker for the 2078 mutation (Asp→Gly) was also used (Neff *et al*., 1998). An A:G mismatch was introduced in the reverse primer to produce a restriction site for EcoRV in the susceptible sequence. The primer pair ACCF1/EcoRV2078r amplified a 353-bp segment of ACCase. EcoRV digestion was performed and homozygous- and heterozygous-resistant and homozygous-susceptible biotype bands were observed. (Table 2-3).

RESULTS

Herbicide Screening Test

Different survival rates to clodinafop-propargyl were identified among the resistant rigid ryegrass biotypes at 28 days after spraying. KHO, HAM2 and AH3

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biotypes were the most resistant based on plant survival rates. As anticipated, the range of resistance was broad for several biotypes, and 16 biotypes (AH3, AH4, AH6, BOS1, BOS2, BOS5, DA1, DA2, DA4, HAM1, HAM2, HAM3, HAM4, HAM5, HAM6, HAM7 and KHO) were characterized as resistant (higher than 20% survival, but many with a high rate of survival). Also, 10 biotypes were characterized as developing resistance (1% to 19% survival), while only three were susceptible to clodinafop-propargyl (Table 4). The resistant biotypes (except for the AH6 and DA4 biotypes, because no seeds were available) were selected for cross-resistance and multiple resistance testing.

Cross- and Multiple Resistance to Herbicides

Cross-Resistance to Commonly Used ACCase-Inhibiting Herbicides

This study identified high rates of resistance to some ACCase-inhibiting herbicides that are widely used in wheat crops. Five of the assayed biotypes displayed greater than 20% survival of diclofop-methyl treatment, eight biotypes had less than 20% survival, and three were susceptible (no survival) to diclofop-methyl (Table 5). Resistance to clodinafop-propargyl and cross-resistance to diclofop-methyl were confirmed in rigid ryegrass during this bioassay. Of the 16 biotypes screened for two ACCase-inhibiting herbicides, 13 biotypes were cross-resistant to both herbicides (Table 5).

Of the 16 rigid ryegrass biotypes, only HAM2 showed more than 20% of the plants surviving sethoxydim treatment and a large majority (15 biotypes) were susceptible to this herbicide (Table 5). Pinoxaden at a dose of 60 g ai ha^{-1} controlled all biotypes (Table 5); however, at a dose of 45 g ai ha^{-1} (recommended rate), one of the tested biotypes had a survival rate greater than 20%, while seven displayed less than 20% survival.

Biotypes AH3, AH4, BOS2, DA2, HAM1, HAM2, HAM3 and HAM4 were able to survive both clodinafop-propargyl and

Table 2. Primers used in the experiment.

Primer	Sequence 5'-3'	Usage	Reference
ACCF1	*CACAGACCATGATGCAGCTC*	CAPS for 2041and 2088	Yu *et al.*, 2007
ACCR1	*CTCCCTGGAGTTGTGCTTTC*	-	
NsiI1781f	*CTGTCTGAAGAAGACTATGGCCG*	dCAPS for 1781	Kaundun and Windass, 2006
NsiI1781r	*AGAATACGCACTGGCAATAGCAGC ACTTCCATGCA*	-	
EcoRV2078r	*GCACTCAATGCGATCTGGATTTATC TTGATA*	dCAPS for 2078	Yu *et al.*, 2007

Table 3. Restriction enzymes used in dCAPS/CAPS analysis.

Enzyme name	Commercial Isomer	Restriction site	Technique	Reference
NsiI	AvaIII, Mph11031	*5´-ATGCAˆT-3´ 3´-TˆACGTA-5´*	dCAPS (1781)	Kaundun and Windass, 2006
EcoRI	FunΠ	*5´-GˆAATTC-3´ 3´-CTTAAˆG-5´*	CAPS (2041)	Yu *et al.*, 2007
*Eco*RV	Eco321	*5´-GATˆATG-3´ 3´-CTAˆTAG-5´*	dCAPS (2078)	Yu *et al.*, 2007
Eco47 ш	AfeI, Aor51HI, FunI	*5´-AGCˆGCT-3´ 3´-TCGˆCGA-5´*	CAPS (2088)	Yu *et al.*, 2007

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Table 4. Comparison of the biotypes of suspected resistance to herbicides Clodinafop-Propargyl using methodology of Owen *et al.* (2007), in southwest of Iran.

Clodinafop-propargyl					
Biotype	Survival % of control [a]	Resistance status [b]	Biotype	Survival % of control[a]	Resistance Status[b]
AH1	17.82 [ei]	DR	DA2	53.62 [ac]	R
AH2	0 [i]	S	DA3	7.12 [gi]	DR
AH3(CAR)	67.85 [ab]	R	DA4	24.95 [eh]	R
AH4	39.25 [ce]	R	HAM1	24.95 [eh]	R
AH5	7.15 [gi]	DR	HAM2	71.43 [ab]	R
AH6	21.5 [ei]	R	HAM3	39.32 [ce]	R
AH7	7.15 [gi]	DR	HAM4	32.20 [cf]	R
AH8	3.57 [hi]	DR	HAM5	25.87 [eh]	R
AH9	3.57 [hi]	DR	HAM6	25.02 [eh]	R
BOS1	24.95 [eh]	R	HAM7	28.57 [eg]	R
BOS2	53.50 [ac]	R	HAM8	10.72 [fi]	DR
BOS3	17.82 [ei]	DR	GOT	3.57 [hi]	DR
BOS4	3.57 [hi]	DR	KHO	75.01 [a]	R
BOS5	25.05 [eh]	R	RAM	0 [i]	S
DA1	39.25 [ce]	R	HF(S)	0 [i]	S

[a] Means presented within each column with no common letter(s) are significantly different according to Fisher's Protected LSD test where $P \leq 0.01$. [b] S= Susceptible; R= Resistant, DR= Developing Resistance**.**

Table 5. The percent of rigid ryegrass biotypes.[a]

City	Clodinafop-propargyl	Clodinafop-propargyl+ oil	Diclofop-methyl	Clethodim	Sethoxydim	Pinoxaden 45 g	Pinoxaden 60 g	Pinoxaden + Clodinafop	Chlorsulfuron	Mesosulfuron + Iodosulfuron	Isoproturon+ Diflufenican	Isoproturon + Diflufenican	Paraquat	Glyphosate
AH3	H	H	H	S	S	L	S	H	H	H	L	S	S	S
AH4	H	L	L	S	S	L	S	S	S	S	S	S	S	S
BOS1	H	H	L	S	S	S	S	S	L	S	S	S	S	S
BOS2	H	H	L	S	S	L	S	S	L	S	S	S	S	S
BOS5	H	H	L	S	S	S	S	S	L	L	S	S	S	S
DA1	H	H	H	S	S	S	S	S	L	S	S	S	S	S
DA2	H	H	H	S	S	L	S	S	L	S	L	S	S	S
HAM1	H	H	L	S	S	L	S	S	H	S	L	S	S	S
HAM2	H	H	H	S	H	H	S	L	H	H	H	S	S	S
HAM3	H	H	L	S	S	S	S	S	L	H	S	S	S	S
HAM4	H	H	L	S	S	L	S	S	S	S	S	S	S	S
HAM5	H	L	S	S	S	S	S	S	L	S	S	S	S	S
HAM6	H	L	L	S	S	S	S	S	S	S	S	S	S	S
HAM7	H	L	S	S	S	S	S	S	S	S	S	S	S	S
KHO	H	H	H	S	S	L	S	S	H	S	S	S	S	S
Susceptible	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TRP	15	15	13	0	1	8	0	2	11	4	4	0	0	0

[a] H: High-resistance (higher than 20% surviving plants); L: Low-resistance (less than 20% surviving plants); S: Fully susceptible (0% surviving plants, (refer to Figure 1 for all herbicides), TRP: Total number of Resistant Biotypes.

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pinoxaden, establishing that cross-resistance extends across the AOPP and PPZ chemical families of ACCase-inhibiting herbicides. For the mixture of clodinafop-propargyl+pinoxaden, only two biotypes (AH3 and HAM2) were able to survive, while the others showed sensitivity to this herbicide (Tables 5).

Of the 15 biotypes of rigid ryegrass that were resistant to clodinafop-propargyl, one biotype was resistant to sethoxydim, eight biotypes to pinoxaden, two biotypes to clodinafop-propargyl+pinoxaden, and one biotype (HAM2) exhibited resistance to all three of the tested ACCase-inhibiting herbicides. Clethodim was the only ACCase-inhibiting herbicide to which no biotypes showed resistance (Tables 5).

Cross-Resistance to ALS Herbicides

This experiment included herbicides belonging to three important ALS-inhibiting families, which are commonly used in the wheat fields of Iran. Twelve of the rigid ryegrass biotypes showed resistance to chlorsulfuron (four resistant biotypes and eight developing resistance) and four had resistance to the ALS-inhibiting herbicides mesosulfuron-methyl+iodosulfuron-methyl (Table 5).

Of the 16 rigid ryegrass biotypes, only the HAM2 had greater than 20% plant survival rate in response to sulfosulfuron+metsulfuron, with three of the 16 rigid ryegrass biotypes developing resistance and 12 biotypes being susceptible to this herbicide. Only three biotypes (AH3, HAM1 and HAM2) were able to survive all three ALS-inhibiting sulfonylurea herbicides used (Table 5).

Twenty-five percent (4 biotypes) of the biotypes screened with the ALS-inhibiting herbicides chlorsulfuron and the mesosulfuron-methyl+iodosulfuron-methyl sodium mixture were cross-resistant to both herbicides.

Resistance to Other Herbicide Groups

All 16 biotypes tested were susceptible to isoproturon+diflufenican, a photosystem II and carotenoid-inhibiting herbicide mixture. In addition, all biotypes were susceptible to the photosystem I-inhibiting herbicide paraquat (Gramoxon; Syngenta) and 5-EnolPyruvylShikimate-3-Phosphate Synthase (EPSPS) glyphosate (Roundup; Bayer) (Table 5).

Multiple Resistance

The results of this study showed that 11 out of the 16 biotypes (69%) of rigid ryegrass showed resistance to both clodinafop-propargyl and chlorsulfuron (Table 5). Fortunately, no resistance was detected to the non-selective herbicides glyphosate and paraquat during this study and no biotypes were resistant to more than two of the herbicide groups (Table 5).

3.3. Molecular Basis of Cross-Resistance to ACCase Herbicides

CAPS Analysis

The Ile-2041-Asn mutation was the most common in the rigid ryegrass biotypes (present as 90% of all observed mutations). Biotype (DA1) had a single band of 492-bp, indicating a homozygous mutation for the resistant 2041-Asn allele. Eight biotypes (AH3, BOS1, BOS5, DA2, HAM1, HAM2, HAM3 and KHO) had three bands at 492, 282 and 208-bp, indicating that they were heterozygous for the resistant 2041-Asn allele. No other biotypes exhibited changes and, therefore, did not contain this mutation (Table 6). The homozygous and heterozygous plants of the rigid ryegrass biotypes treated at the recommended rate of clodinafop-propargyl survived, while the Susceptible (S) plants died. The substitution of Cytosine (C) with Thymine (T) at codon 2088 causes an amino acid substitution of cysteine with arginine and creates an $Eco47_{III}$ restriction site. After digestion with $Eco47_{III}$ enzyme, all of the biotypes had the wild type digestion pattern meaning that a

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Table 6. Genotyping of different clodinafop-propargyl resistant and susceptible *Lolium rigudum* biotypes from Iran, to detect specific point mutations in ACCase gene (= No mutation) by using the CAPS and dCAPS techniques.

Biotype	Resistance status	Mutations analyzed			
		Ile-1781 ATT	Ile-2041 ATT	Asp-2078 GAT	Cys-2088 TGT
AH3	Resistant	=	AAT	=	=
AH4	Resistant	=	=	=	=
BOS1	Resistant	CTT	AAT	=	=
BOS2	Resistant	CTT	=	=	=
BOS5	Resistant	=	AAT	=	=
DA1	Resistant	=	AAT	=	=
DA2	Resistant	=	AAT	=	=
HAM1	Resistant	=	AAT	=	=
HAM2	Resistant	=	AAT	=	=
HAM3	Resistant	=	AAT	=	=
HAM4	Resistant	=	=	=	=
HAM5	Resistant	=	=	=	=
HAM6	Resistant	=	=	=	=
HAM7	Resistant	=	=	=	=
KHO	Resistant	=	AAT	=	=
HF (S)	Susceptible	=	=	=	=

mutation at 2088 of the ACCase enzyme was not present (Table 6).

dCAPS Analysis

One mutation in ACCase was observed at Ile-1781-Leu for two biotypes. These two biotypes (BOS1 and BOS2) were homozygous for the resistant Ile-1781-Leu allele, but had no mutation at 2078 (Table 6).

DISCUSSION

Resistance to ACCase-inhibiting herbicides and other herbicide groups is an emerging problem in Iran. The results of the current study have revealed the first rigid ryegrass biotypes to be resistant to both ACCase- and ALS-inhibiting herbicides in Iran. The first case of resistance to ACCase-inhibiting herbicides in Iran was observed in wild oat in southwestern Iran, in 2006 (Zand *et al.*, 2006). Many years after this report, statistics showed the spread of weed resistance to different herbicides in more weed species (Gherekhloo *et al.*, 2016).

The whole-plant assay clearly showed that the 27 biotypes were developing resistance or were resistant to clodinafop-propargyl. Weed resistance to ACCase-inhibiting herbicides has been previously reported for several grass species, such as Italian ryegrass (*L. multiflorum*) (Stanger and Appleby, 1989; De Prado *et al.*, 2000), rigid ryegrass (*L. rigidum*) (Llewellyn and Powles, 2001), wild oat (*A. fatua* L. and *A. sterilis* L.) (Heap *et al.*, 1993; Travlos, 2013), rigid brome (*Bromus rigidus* Roth), little canarygrass (*P. minor*) (Tal *et al.*, 2000), and green foxtail (*Setaria viridis*) (De Prado *et al.*, 2004).

Despite the high number of biotypes that were resistant to ACCase- and ALS-inhibiting herbicides (about 69% of biotypes studied), crop rotation was infrequent and wheat monoculture was the rule in these areas. The rotation of herbicides was an unknown practice, further increasing the selection pressure for resistance. The extended use of ACCase- and ALS-inhibiting herbicides in Iran is due to their efficacy against a large number of other

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weed species, such as *A. ludoviciana*, *Bromus* spp., *Hordeum* spp. in wheat, resulting in ongoing selection pressure in rigid ryegrass. As a result, biotypes of rigid ryegrass with multiple resistance to certain ACCase- and ALS-inhibiting herbicides have already emerged. Currently, multiple resistance to ACCase- and ALS-inhibiting herbicides has been confirmed in other weed grass species, such as *A. ludoviciana*, *Bromus* spp. and *Hordeum* spp. (Boutsalis *et al.,* 2012; Owen *et al.,* 2012a; 2012b).

In the rigid ryegrass biotypes that were reported as having resistance to ACCase, target site and non-target site mechanisms can occur (Preston and Mollary-Smith, 2001; Farzaneh *et al.,* 2015). Studies indicate that target site mutations are a common mechanism of providing resistance to ACCase-inhibiting herbicides in grasses in Iran and rigid ryegrass in South Australia (Zand *et al.,* 2006; Malone *et al.,* 2010). Other studies on ACCase-resistant *A. myosuroides* in France have reported resistance without mutation in ACCase (Menchari *et al.,* 2006 and 2007; Délye *et al.,* 2007). The current study showed that two mutations led to resistance to ACCase-inhibiting herbicides in resistant rigid ryegrass biotypes, an Ile-2041-Asn mutation (in eight biotypes) and an Ile-178-Leu mutation (in two biotypes). Homozygous mutants for the 1781-Leu allele can confer resistance on the recommended rates of clodinafop-propargyl, which is similar to other studies (Yu *et al*. 2007; Kaundun and Windass, 2006).

The substitution of leucine for isoleucine in the 1781 position is one key point mutation that increases resistance to different AOPP herbicides in weed grass species (Délye *et al.,* 2003 and 2005; Preston, 2009; Powles and Yu, 2010; Zand *et al.,* 2013; Vila-Aiub *et al.,* 2015). Malone *et al*. (2010) reported that the 2041-Asn mutation in rigid ryegrass was more common than the 1781-Leu mutation and a larger number of resistant biotypes showed this mutation. The results of this study showed that the mutation at the Ile-2041 point was most common for the rigid ryegrass resistant biotypes in southwestern Iran.

Previous surveys of wild oats and rigid ryegrass in Iran found the major mutations in ACCase to be at Ile-1781-Leu (Zand *et al.,* 2009) with mutations at 2041-Asn accounting for less than 10% of the mutant alleles identified. From 2006 to 2015, there was an increase in the number of grasses in Iran with resistance to ACCase. The number of amino acid substitutions at 1781-Leu decreased, while the number of amino acid substitutions at 2041-Asn increased. The amino acid modification at 2041-Asn appears to result in a high level of resistance to clodinafop-propargyl and other herbicide groups, which may be why it is becoming more common.

CONCLUSIONS

Two ACCase mutations (1781-Leu and 2041-Asn) were identified in 10 rigid ryegrass biotypes that were resistant to clodinafop-propargyl and diclofop-methyl. Moreover, the mutations with homo/heterozygous 2041-Asn and the homozygous 1781-Leu confer a sufficient level of resistance to ACCase- herbicides. The results of this study conclusively demonstrate that resistance to ACCase- and ALS-inhibiting herbicides has occurred in many rigid ryegrass biotypes in the wheat fields of Khuzestan Province, Iran. The use of herbicides has become the most common weed control method in wheat fields in Iran and ACCase- and ALS-inhibiting herbicides have been used for more than 15 years. Unless farmers improve and diversify their weed management methods, resistance to ACCase- and ALS-inhibiting herbicides will further expand and become a major problem in this province in the near future. However, realistic opportunities exist for the reduction of selection pressure against resistant biotypes by means of crop and herbicide rotation and other agronomic practices, which would provide the crop with a

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competitive edge over the weeds. These methods include early crop sowing, competitive cultivars, and increased seeding rates.

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مقاومت عرضی و چندگانه علف هرز چچم یک‌ساله (*Lolium rigidium* Guad.) به علف‌کش‌ها در ایران

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چکیده

رقابت علف‌های هرز، به‌ویژه گونه‌های باریک برگ، تخمین زده می‌شود که باعث کاهش ۲۳ درصدی عملکرد محصول در مزارع گندم ایران می‌شود. در طول سال‌های ۱۳۹۲ تا ۱۳۹۵، مطالعه‌ای برای ارزیابی مقاومت ۳۰ بیوتیپ علف هرز چچم یک‌ساله به علف‌کش‌ها که از مزارع گندم استان خوزستان جمع‌آوری‌شده بود، انجام شد. غربالگری این بیوتیپ ها با استفاده از علف‌کش کلودینافوپ پروپارژیل در گلخانه انجام شد و درنهایت بیوتیپ هایی که پس از کاربرد علف‌کش بیش از ۲۰ درصد افراد زنده داشتند برای تائید مقاومت عرضی و چندگانه موردبررسی قرار گرفتند. به ترتیب ۹۴ و ۷۵ درصد بیوتیپ های چچم یک‌ساله به علف‌کش‌های بازدارنده استیل کو آنزیم آ کربوکسیلاز (ACCase) و استولاکتات سینتاز (ALS) مقاومت نشان دادند. همچنین شایان‌ذکر است که تقریباً ۶۹ درصد از بیوتیپ‌های چچم یک‌ساله دارای افرادی مقاوم به علف‌کش‌های با دو مکانیسم عمل متفاوت بودند. بر اساس این نتایج، این اولین گزارش درباره‌ی مقاومت‌های عرضی و چندگانه در بیوتیپ های چچم یک‌ساله از ایران است. برگ‌های گیاهان چچم دارای مقاومت عرضی نسبت به علف‌کش‌های بازدارنده‌ی ACCase با استفاده از مارکرهای CAPS و dCAPS، برای شناسایی جهش‌های احتمالی در موقعیت‌های ۲۰۴۱، ۲۰۸۸، ۱۷۸۱ و ۲۰۷۸، آنالیز شدند. به ترتیب در ۲ و ۹ بیوتیپ جهش در موقعیت‌های ۱۷۸۱ و ۲۰۴۱ مشاهده شد و این جهش‌ها باعث اعطای مقاومت به علف‌کش‌های بازدارنده ACCase پلاستیدی گردیدند. این نتایج نشان می‌دهد که روند فعلی مقاومت علف‌کش‌ها در چچم یک‌ساله با مشکلات جدی روبه رو می‌باشد و با توجه به مقاومت عرضی و چندگانه، لازم است راهکارهای مدیریت تلفیقی در درازمدت، اجرا شود.

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