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Resistance of various biotypes of Canary grass (*phalaris. Spp*) to acetyl-CoA carboxylase-inhibiting herbicides

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Abstract

Little seed canary grass (*Phalaris minor* L.) is a major weed in wheat fields in some parts of Iran. To evaluate the efficacy of molecular and greenhouse methods in detecting the resistance of 49 biotypes of canary grass(*Phalaris. Spp)* to acetyl-CoA carboxylase-inhibiting herbicides, two methods including whole plant screening and PCR-based molecular methods were applied. Results showed that there were resistant biotypes (ile-1781-Leu) among the studied weed populationand the similarity between greenhouse and molecular methods was 67%. According to the molecular method, an isoleucine (ile) 1781 to leucine (leu) mutation in plastidicACCase enzyme of 30 biotypes (67% of biotypes) was identifiedas a mutation endowing to the clodinafop-propargyl resistance. The partial differences of about 33% between greenhouse and molecular methods can be explained by mutation in another location or through another metabolism – based mechanism.

Key words: ACCase inhibitors; resistance;*Phalaris sp*.;whole plant assay; PCR

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Introduction

Weed resistance to herbicide is a universal phenomenon. There are 429 weed species (222 dicotyledon and 207 monocotyledon) in 80 various crops which have become resistant to 22 distinct herbicide groups and 153 herbicides in 65 countries (Heap, 2014). Approximately 61 % of the total resistant speciesbelong to ALS inhibitors(72 species), PSII inhibitors(144 species), and ACCaseinhibitors (45 species)(Heap,2014). Resistance of narrow leaf weeds to herbicides is such a problematic issue that has caused a serious threat to sustainable production of agricultural crops. Although, narrow leaf weeds contribute to 25% of resistant species, they comprise about 40% of resistant biotypes (Beckie, 2007).

Since their introduction in the 1970s and 1980s (Delye, 2005), the increase in the usage of graminicides belonging to the ACCase inhibitors, has led to a parallel increase in the evolution of

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populations resistant to these herbicides (Tal *et al*., 2000). During the past decades there has been an increasing number of reports on weed resistance to graminicide (De Prado *et al*., 2000). Heap (2010) reported graminicide resistance in 38 grass weed species worldwide. Grass weed resistance to ACCase inhibitors is steadily increasing world-wide (Heap, 2007). The first case of a resistant grass weed was documented in *P. minor* Retz. at the Gilat Experimental Station in the Negev Desert (Tal *et al*., 1996). There are several reports of *Phalarisspp* resistance to herbicides. In India, this weed has shown resistance to urea and amid herbicidesit showed resistance to Fenoxaprop – p – ethyl(Bor*etal.,*1973; Tharayil-Santhakumar*et al*.,2003). In the U.S. and Australia it showed resistance to ACCase inhibitors while in South Africa resistance to ACCase and ALS inhibitors was observed(Camper,2005). Narrow leaf weeds' resistance to ACCase inhibitors was also reported by Zand*et al*., 2006). They reported resistance of wild oat, lolium, and canary grassin some provinces of Iran and stated that continuous application of herbicides with similar mode of action has caused such a resistance.

ACCase inhibitor herbicides are among efficient post-emergence herbicides applied to narrow leaf weeds in cereal. The most common approach to control *P. paradoxa* in cereal crops is through application of acetyl-CoA carboxylase (ACCase)-inhibiting herbicides. These compounds which are also categorized as class A herbicides (HRAC, 2008), consist of three chemical groups, namely,cyclohexanediones (DIMs), aryloxyphenoxypropionates (FOPs), and phenylpyrazolines (DENs; e.g. pinoxaden). These herbicides block activation of ACCase, which is a key enzyme in catalyzing the initial step in fatty acid biosynthesis (Buchanan *et al*., 2000). There are two isoforms of ACCase in plants: the plastid-localized ACCase, which is essential in the biosynthesis of primary fatty acids, and the cytosol-localized ACCase, which is involved in biosynthesis of long-chain fatty acids (Yu *et al*., 2007). All isoforms of ACCase have three catalytic domains:the biotin carboxyl-carrier (BCC), the biotin carboxylase (BC), and carboxyl transferase (CT) (Nikolau et al., 2003; Delye and Michel, 2005; Liu *et al*., 2007). The CT domain of the plastid-localized multi-domain ACCase is the target of action for ACCase-inhibiting herbicides, and any changes in the structure of this domain are responsible for insensitivity of the enzyme to these herbicides (Zhang *et al*., 2004; Yu*et al*., 2007). Resistance to ACCase inhibitors in narrow leaf weeds is due to three known mechanisms, namely, enhanced metabolism, over-expression of ACCase, or the presence of an altered, insensitive form of the plastidicACCase enzyme (Brown *et al*., 2002).

At present, *P. minor* is one of the two most important grass weeds in wheat and barley fields in Iran (MinbashiMoeini*et al*., 2000), drastically affecting crop yield. To control the grass weeds in these crops, some ACCase inhibitors have been registered in Iran over the last three decades. Recently, two *P. minor* populations have been found in wheat fields of Fars province in the south of Iran which are resistant to almost all APPs that are commonly used in these fields (Gherekhloo*et al*., 2011). This is not surprising, as these three herbicides have continuously been applied at least for the last two decades in wheat and barley fields of Fars province. There are several methods for identificationof weed resistance to herbicides including bioassays of seedling (Corbet andTardif, 2006), petri dish assay(Cirujeda*et al*., 2001), pollen test (Boutsalis,2001), leaf fragment evaluation(Patzoldt and Tranel,2002), and enzyme assay(Corbet andTardif,2006). Despite their high accuracy, all methods mentioned above require broad space for performance and fail to recognize the cross-resistance and mechanism of resistance (Corbet andTardif,2006;Koundan and Windass,2006;Delye*et al*.,2002). There are many studies in which DNA- based methods, as powerful and simple methods, have been employed to detect weed resistance (Kaundan andWindass,2006). Kaundan andWindass (2006) used PCR-based molecular method to identify mutation site in narrow leaf weeds resistant to ACCase inhibiting herbicides. They believe that molecular method is a simple, potent, and cost effective method that is applicable to different species and is capable of recognizing both homozygote (Leu/Leu1781) and heterozygote (Ile/Leu1781) amino acid substitution in ACCase

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Table 1
Name and collection areas of collected little seed canary grass biotypes

Biotype	Collection area	Biotype	Collection area
S2/ 84/RR	Khouzestan	G10/85	Golestan
SH7/84	Khouzestan	G3/85	Golestan
A3/84	Khouzestan	G11/85	Golestan
AN/84	Khouzestan	G/S/86	Golestan
A4/84	Khouzestan	G7/85	Golestan
ES/85	Fars	G2/85	Golestan
M1/86	Fars	G1/2 86	Golestan
F2/86	Fars	G9/86	Golestan
M/85R	Fars	G8/86	Golestan
ES3/86	Fars	G10/86	Golestan
J2/86	Fars	G5/86	Golestan
SH2/85	Fars	G4/86	Golestan
F2/85R	Fars	G3/86	Golestan
sh2/85/RRR	Fars	G6/86	Golestan
F4/85RRR	Fars	G4/85	Golestan
F3/86	Fars	G7/85	Golestan
ES1/85	Fars	G5/85	Golestan
M2/85R	Fars	G1/86	Golestan
F/85S	Fars	G7/86	Golestan
FI2/86	Fars	G9/85	Golestan
F/K/86	Fars	kh/s1/84	Khouzestan
F/M2/86	Fars	AS/90	Khouzestan
J1/86	Fars	SHT/84	Khouzestan
SH1/85	Fars	A2/84	Khouzestan
-	-	D/84	Khouzestan

enzyme and it provides a basis for accurate measurement of dominant allele frequencies in specific populations(Kaundan andWindass, 2006). Delyeet al. (2002) studied the resistance of foxtail and lolium to ACCase inhibitor herbicides and stated that the isoleucine (ile) to leucine (lue) substitution at the position of 1741(Ile/Lue) was the most common amino acid substitution in both weeds. Zand*et al.* (2009) studied the resistance of wild oat, canary grass, and rye grass to clodinafop-propargyl in some provinces of Iran and found that an Ile/Leu1781mutation is the most common resistance-endowing mutation to these herbicides.In this research the efficiency of greenhouse and molecular method to detect the resistance of 49 various biotypes of *Phalaris minor*to Clodinafoppropargyl were compared.

The main objectives of the present study were (i) to study the mechanism behind resistance to ACCaseinhibiting herbicides; (ii) to determine the molecular basis for resistance to ACCase inhibitors, and (iii) to compare efficiency of glasshouse and molecular methods for detectionof resistance of various biotypesof *Phalaris minor* to Clodinafoppropargyl.

Materials and Methods

Plant material

Forty nine little seed canary grass biotypes were collected from Fars, Khoozestan and Golestan provinces(Table 1).

Glasshouse experiment(seedling screening)

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An experiment was conducted in the greenhouse facilities of the Department of Weed Research at the Iranian Research Institute of Plant Protection. The seeds of various canarygrass biotypes were planted in small pots and as a CRD

Table 2
Sequences and names of primers used in PCR(Delye et al., 2002)

Primer name	Primer Sequences 5'-3'
VRDIC+	5'-GGA CTA GGT GTG GAG AACC-3'
VRDITR	5'-CAA TAG CAG CAC TTC CAT GTA A-3'
ACVRG1	5'-AAT GGG TCG TGG GGC ACT CCT
ACVRG1R	5'-GCT GAG CCA CCT CAA TAT ATI AGA AACACC-3'

design with three replications. Each replication comprised one pot and there was also one control pot per replication. Irrigation was applied throughout the experiment to prevent drought stress. Seedlings were sprayed with 0/8 L/ha clodinafoppropargyl at two- to four-leaves stage. The number of living seedlings was recorded one month prior to and after spraying with herbicide and the percentage of living seedling following herbicide application was calculated. The living seedlings were cut from the ground, oven –dried at 75° C for 48 hours and weighed. Mean dry weight of single seedling per treated biotype compared to the control, was obtained based on the number of seedlings and aerial dry weight of total seedlings per pot. Four weeks after herbicide spraying, different plant attributes such as numbers, fresh and dry weights, and percentage of reductions were calculated. The number of living seedlings, the number of dead seedlings, fresh weight, dry weight, percentages of fresh and dry weights compared to the control, viability percentage in comparison to the control, mean fresh weight, mean dry weight, fresh weight reduction and dry weight reduction, were calculated based on methods of Mous- et al. (2007) and Adkins *et al*. (1997).

DNA extraction

DNA isolation was carried out using CTAB method (Cullinges, 1992) following the protocol. CTAB buffer (2%) was prepared using 10ml of Trise-Hcl100Mm (pH=8), 4ml of EDTA20mM (pH=8), 28ml of NaCl 1/4M, and 1g of PVP. To isolate DNA, 0.2g of plant leaf tissue was grinded with liquid nitrogen and 800µl of CTAB buffer was added to the extract and then the tubes were stored at water bath for 1 hour at 55°C. The tubes were shaken every 10 minute. Equal volume of chloroform-isoamylalchol (24:1) was added to each tube and the mix was gently shaken for 10 minutes. Tubes were centrifuged at 13000g for 10-15 minutes and supernatant was transferred to another tube. Equal volume of isoamylalchol was added to each tube and tubes were then gently mixed for several times and subsequently stored at -20°C for 20 minutes. In the next step , tubes were centrifuged at 13000g for 5 minutes and supernatant was discarded . DNA plate was washed with 70% ethanol and dried at room temperature. At the end , 60µl double distilled water was added to each tube and stored at -20°C (Cullings, 1993). The quantity and quality of isolated DNA was determined by biophotometer system.

Polymerase Chain Reaction (PCR)

PCR amplification was performed using thermo cycler system (Bio Rad) in total volume of 30µl. Each reaction contained 0.1µl of each primer, 1µl $MgCl_2$, 0.5µl d NTP mix, 1.5µl of sample DNA, 0.2µl Taq polymerase (5unit), and distilled water. The PCR program contained denaturing at 94° C for 1.5 minute followed by 35 cycle amplification where each cycle involved three separate stages (denaturing at 94°C, annealing at 55°C for 30 seconds and extension at 72°C). The final extension was performed at 72°C for 10 minutes.

Analysis of PCR results

To evaluate the resistance or sensitivity of biotypes, PCR amplification was performed by specific primers. Primers were designed based on mutation occurrence (substitution of A to T or C, Ile-1781-Leu), and analysis of results and determination of mutation mechanism were

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performed according to the size of amplified fragment in sensitive and resistant biotypes (Delye*et al*., 2002).

Results

Seedling screening through herbicide treatment

Results of herbicide screening showed that all biotypes except G10/86 were resistant to clodinafoppropargyl in Golestan province. In case of the sensitive biotype, the number of living phalaris seedlings was 52% of that prior to herbicide treatment. Fresh and dry weights of single seedling compared to control were 17% and 27%, respectively (Table3). These results showed that herbicide application could not decrease the number of them before treatment but it could dramatically reduce the dry and fresh weights. According to Adkins et al. (1997), biotypes with more than 50% survival and 80% dry weight of control treatment are grouped as resistant. According to this criteria, 17 biotypes (out of 20 biotypes), were identified as resistant biotypes in this province. In case of khouzestan biotypes, results indicated that all biotypes except for Kh/s1/84and AN/84 were resistant (Table 4). At Fars, only one biotype (ES/85) showed sensitivity to clodinafop-propargyl(Table 5).

Table 3
Results of herbicide screening for Golestan biotypes

Biotype	Percentage of Living Seedlings(%)	Fresh Weight Reduction(%)	Dry Weight Reduction(%)
G10/85	57.93	44.45	50.19
G3/85	53.15	36.37	63.2
G11/85	82.87	37	43.42
G/S/86	68.05	50.7	69.07
G7/85	59.46	36.51	50.73
G2/85	50	62.51	51.22
G1/2 86	24.72	63.71	47.55
G9/86	100	24.45	80.91
G8/86	60.83	35.04	56.47
G10/86*	52.22	82.42	27.88
G5/86	53.33	35.91	56.64
G4/86	100	11.04	84.49
G3/86	24.5	73.65	32.05
G6/86	56.54	51.1	54.09
G4/85	57.5	41.98	52.37
G7/85	55.55	42.63	42.88
G5/85	86.11	17.08	59.99
G1/86	30.35	54.14	45.05
G7/86	56.11	41.62	43.48
G9/85	68.05	44.12	31.84

PCR-based molecular method and evaluation of Ile-leu substitution

Substitution of A to C or T nucleotide in the position of 5341 of ACCase enzyme resulted in substitution of Ile-1781-Leu isoleucine to leucine in the ACCase enzyme. This mutation was identified as the reason of resistance of some narrow leaf weeds to ACCase inhibitor herbicides. To distinguish the resistant plants, PCR amplification with specific primers was performed based on Delye et al. (2002). Primer pairs of ACVRG1 and ACVRG1R produced a 785

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bp band that was amplified by all ACCase alleles (internal positive control). Amplification with VRDITR andACVRG1 showed the presence of an ACCase allele with T nucleotide at 5431 that amplified a 495bp fragment. Amplification with ACVRG1RandVRDIC+ showed an ACCase allele with a C nucleotide at the position of 5431 that produced a

Table 4
Results of herbicide screening for Khouzestan biotypes

Biotype	Percentage of alive seedlings(%)	fresh weight reduction(%)	Dry Weight Reduction(%)
kh/s1/84*	41.11	84.83	46.66
AS/90	56.54	51.32	60.09
SHT/84	48.21	57.22	74.57
A2/84	47.61	60.01	43.81
D/84	57.5	28.31	75.05
S2/ 84/RR	54.16	47.09	61.01
SH7/84	89.16	14.92	79.3
A3/84	64.58	36.67	81.24
AN/84*	17.88	80.87	36.77
A4/84	63.55	43.85	77.45
ES1/85	57.5	7.75	85.63
M2/85R	31.94	54.17	56.01
F/85S	49.16	51.42	68.19
FI2/86	79.44	20.76	75.92
F/K/86	76.94	41.5	78.65
F/M2/86	71.3	90.65	64.75
J1/86	60.47	23.93	76.75
SH1/85	95.23	11.12	72.95
ES/85 *	50	85.01	38.71

329bp fragment after amplification. Sensitive biotypes only showed the 785bp fragment and resistant biotypes showed both 329bp and 495bp based on mutation occurred at position 5341.Biotypes with three amplified fragments, belonged to resistant biotypes and T or C nucleotide was substituted by A nucleotide. In the present study, 30 biotypes out of 49 biotypes were resistant to clodinafop-propargyl. this research, the primers which were used in PCR reaction were designed based on mutation occurred at 5341 position of ACCase enzyme. This observed mutation is the most common mutation endowing resistance to ACCase inhibiting herbicides in phalaris and other narrow leaf weeds. In our study, the primers detected 67% of biotypes as resistant. Delye*et al*. (2002) studied 1800 foxtail, and 705 ryegrass biotypes, and detected 508 and 271 resistant biotypes in these two species to ACCase inhibiting herbicides, respectively (Delye*et al*, 2002). In the present research, there were some biotypes that were identified as sensitive according to the greenhouse screening, which may indicate that mutation in other positions or other resistance mechanisms such as enhanced metabolism may be responsible for that (Zand, 2010). Zand (2010) evaluated the resistance of wild oat, phalaris and ryegrass to ACCase inhibiting herbicides and found that mutation at position 1741 or substitution of leucine by isoleucine has been the reason of occurrence of resistance in 44% of evaluated biotypes(Fig. I).

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Comparison of molecular method with greenhouse method

Based on greenhouse method, 4 biotypes out of the 49 evaluated biotypes were detected as sensitive. However, molecular method revealed 30 resistant biotypes from the same biotypes population. Comparison of between the two methods showed that if the biotypes that were detected as semi-resistant by the greenhouse method, are grouped as resistant biotypes based on Mouse*t al*. (2007), then the similarity between the two methods would be 67%. The inconsistency between the two methods could be attributed to possible mutations at other positions other than Ile1781-Leu and/or none target site resistance (enhanced metabolism)(Delye, 2005).

Discussion

There are various mechanisms involved in the resistance occurrence to ACCase inhibiting herbicides, which could be summarized as follows: *a*) existence of insensitive target enzyme in resistant species that occurs through a simple co-dominant mutation in gene encoding the ACCase. *b*) mutation in the cell membrane of resistant species that causes repolarization of cell membrane after induction of nonpolarisation as a result of application of ACCase inhibiting herbicide. *c*) the differences in herbicide metabolism in resistant species (Kandan and Windass, 2006; Delye and Mishel, 2005). Despite the various resistance mechanisms mentioned above, the insensitivity of target enzyme to this group of herbicides is known as the most common mechanism (Kandan and Windass, 2006; Delye and Mishel, 2005). There are 11 mechanisms of mutation that confer resistance to ACCase inhibiting herbicides (Bekie, 2012) . To determine the mutations mentioned above, we should use special primers. Since the Ile1781-Leu substitution is the most common mutation (Preston, 2003), the primer which was used in this study, is the most efficient primer to detect resistance using the molecular method. Preston (2003) stated that the Ile1781-Leu substitution and Trp2027-Cys are the most common

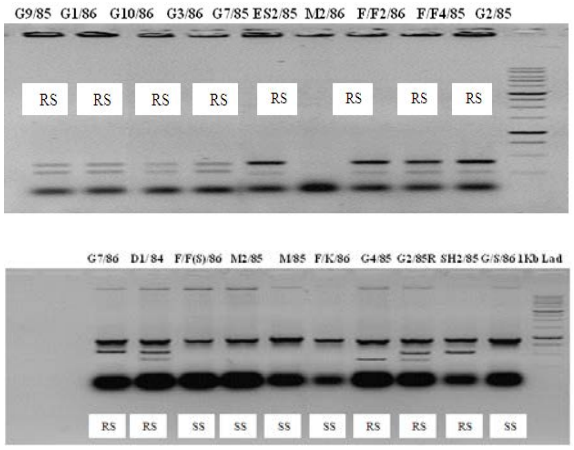

Fig. I.CR amplification using specific primers; RS shows resistant biotypes. SS shows sensitive biotypes.

mechanisms of resistance in resistant biotypes and stated that there was no obvious reason for this issue. In general, comparison of the two methods indicates that it is possible to detect the resistance of weeds to ACCase inhibitor herbicides with a negligible error. Today, one of the fastest and most accurate methods for detection of resistant biotypes is DNA-based detection (Corbet and Tardiff, 2006). This method, however, is only able to detect resistance mechanisms as a result of target site modifications, which indicates a constraint of this method for not being able to detect non-target site modification(Corbet and Tardiff,2006;Delye,*et al*,2002). Studies have shown that although the resistance occurrence to the ACCase inhibiting herbicides may arise from enhanced metabolism, but the most common mechanism of resistance occurrence to this group of herbicides is target site alteration-based resistance that happen through the mutation in genes encoding ACCaseenzyme (Delye and Michel, 2005). As mentioned earlier, the inconsistency between molecular and greenhouse methods may be attributed to mutations in other loci (except Ile1741Leu).Therefore, to develop DNA-based identification techniques, more accurate complementary experiments are essential to study and identify target site mutations in other loci.

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بررسی مقاومت بیتوپ های مختلف فالاریس(phalaris. Spp)به علف کش های بازدارنده استیل کوآنزیم آ– کربوکسیلاز(ACCase)

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چکیده فارسی

فالاریس یکی از مهمترین علف های هرز مزارع گندم در برخی نواحی ایران می باشد .به منظور بررسی کارایی روش های مولکولی و گلخانه ای در تشخیص مقاومت ۴۹ بیوتیپ فالاریس به علفکش های بازدارنده استیل کوآنزیم آ- کربوکسیلاز ، دو روش شامل غربالگری گیاهچه کامل در گلخانه و روش مولکولی مبتنی بر PCR بکار گرفته شد.نتایج نشان داد که در بین بیوتیپ های مورد مطالعه ، بیوتیپ های مقاوم (Ile-1781-Leu) وجود داشت که درصد مشابهت دو روش مولکولی و گلخانه ای ۶۷درصد بود.مطابق روش مولکولی ، جانشینی ایزولوسین به جای لوسین در کدون شماره ۱۷۸۱ در آنزیم ACCase پلاستیدی در ۳۰ بیوتیپ(۶۷درصد بیوتیپ ها) از ۴۹ بیوتیپ مورد مطالعه شناسایی شد و نتایج نشان داد که مشابهت بین دو روش مولکولی و گلخانه ای ۶۷ درصد بود.بر طبق روش مولکولی ،جانشینی ایزولوسین به جای لوسین در موقعیت کدون ۱۷۸۱ در اثر موتاسیون، به عنوان عامل بروز مقاومت به کلودینافوپ پروپارژیل شناسایی شد . تفاوت جزئی بین نتایج روش مولکولی و روش گلخانه ای (۳۳درصد) را نیز میتوان به موتاسیون در موقعیت های دیگر یا مکانیسم های تغییر در متابولیسم مربوط دانست.

کلمات کلیدی: مهار کننده های ACCase ،مقاومت،فالاریس،سنجش گیاه کامل،PCR

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