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HERBICIDE RESISTANCE IN ANNUAL RYEGRASS (*Lolium rigidum* Gaud.) POPULATIONS IN WHEAT FIELDS OF FIROOZABAD IRAN

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ABSTRACT

An experiment was conducted to detect herbicide resistance in ryegrass (Lolium rigidum Gaud.) populations infesting wheat (Triticum aestivum L.) fields across the Firoozabad city, Fars Province, during 2008-09. Ryegrass population in each agronomic zone was sprayed with several herbicides having different modes of action using upper recommended dose. Some of these ryegrass populations were found to be resistant to the ACCase–inhibitor herbicides: diclofop-methyl 60% and clodinafop-propargyl 50%. These populations were completely susceptible to herbicides with other modes of action like ALS-inhibitors, mesosulfuron+iodosulfuron, tubuline formation inhibitors, trifluralin and phenylpyrazolin, and pinoxaden. We did not detect any Point mutation 2078-Gly in ryegrass populations through the molecular dCAPS approach.

Keywords: Cross resistance, dCAPS, mutation, ryegrass.

INTRODUCTION

Evolved herbicide resistance is affected by the genetic, weed biology, herbicide mode of action and application methods. In conventional agriculture, weed control is based on herbicide use. Worldwide, Herbicide resistance is an increasing concern, and more weed species have developed resistance to group B herbicides than to any other mode of action group (Mcgillion and Storrie, 2006). Rates of spontaneous mutation vary from organism to organism and from locus to locus, ranging from 7-10 mutations per base pair per replication in some bacteriophages to 10-11 per base pair per replication in humans (Drake *et al.,* 1998). Recently, researchers have identified molecular mutations in the ACCase gene endowing target site based herbicide resistance in some ACCase herbicide-resistant *Lolium rigidum* Gaud. populations. The aim of this research was to test some of the ACCase herbicides in controlling annual ryegrass resistant biotype and its spread. This study also examined the target site 2078-Gly resistance

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to diclofop-methyl and other ACCase-inhibiting herbicides in populations of *L. rigidum* by use of dCAPS method.

MATERIALS AND METHODS

Crop fields were surveyed over a three-week period just before the 2009 wheat grain harvest. Crop fields in three regions (A, high use of single mode of action herbicides, B, medium and C, low) were randomly visited. Fields were surveyed by two people walking in an inverted 'V' 100m into the crop. Fully mature annual ryegrass spikes were collected and bulked from mature plants in the sampling path. Immediately after collection, the ryegrass seed samples were stored in a non air-conditioned glasshouse over summer to break seed dormancy, ensuring maximum germination (Steadman *et al*., 2003). The methods described are similar to those used in the 1998 survey (Llewellyn and Powles, 2001).

Resistance Testing

During the 2010 growing season, 50 seeds of each of the 42 ryegrass populations were planted in plastic seedling trays containing peat. Trays were kept outdoors at the University of Firoozabad, Iran and were watered and fertilized as required. For almost all populations, germination and seedling emergence was high (>90%), ensuring that 40 individual seedlings in each population were screened to each herbicide. Seedlings were treated with herbicide (upper recommended field rates) using an 8002 Tee-jet nozzle sprayer delivering herbicide in 200 L ha^{-1} water at 210 kPa. Known resistant and susceptible ryegrass populations were used as controls for each herbicide treatment. In all experiments, 100%mortality occurred in the known susceptible population.

Table-1. Herbicides and adjuvants applied to ryegrass populations during 2010.

Chemical Class	Common name	Formulation	Rate / Adjuvants
Aryloxyphenoxy-propionate	diclofop-methyl	36% EC	3 lit. ha^{-1} cytogate 0.2%
Aryloxyphenoxy-propionate	clodinafop-propargyl	8% EC	1.5 lit. ha^{-1} cytogate 0.2%
Phenylpyrazolin	pinoxaden	10% EC	600ml ha^{-1} Adigore 0.5%
Sulfonylurea	mesosulfuron+iodosulfuron	30%+30% WG	500g ha^{-1} cytogate 0.2%
Triazines	atrazine	80% WP	1500g ha^{-1}

Herbicide effect was assessed by determining seedling mortality 21 days after herbicide treatment. Ryegrass populations were classed as resistant if 20% or more of the individuals in the population survived the herbicide. Where there was 1–19% survival, the population was classed as developing resistance. Where there was less than 1% survival, the population was classed as susceptible. (Tardif and Powles, 1994). Seedlings that survived diclofop-methyl at 21 days after treatment were cut back to a height of 20 mm, allowed to regrow for 1 week, and then treated with the sethoxydim 12.5%EC (2 L ha^{-1}). Sethoxydim was applied to diclofop-methyl survivors because previous work showed that although ryegrass can metabolise diclofop-methyl, it cannot metabolise sethoxydim (Tardif and Powles, 1994). Therefore, resistance to sethoxydim indicates target site resistance.

Detection of 2078-Gly Mutation

Fresh leaf material (~1cm^2) was harvested from young leaves of a single resistant plant for each resistant population; snap frozen in liquid nitrogen and stored at 80°C. DNA was extracted using the cetyl terimethyl ammonium bromide (CTAB) (Doyle *et al*., 1987). The concentration of nucleic acids was determined spectrophotometrically on a NanoDrop ND-1000 (Thermo scientific, USA) at 260 nm. The PCR was conducted in a 25 mL volume that consisted of about 300 ng of genomic DNA, 0.5 mM of each primer, and 12.5 mL of Taq polymerase (Cinagen, Iran). The PCR reaction was run in a (Techne, UK) with the following profile: 94 ^{o}C 4 min, 35 cycles of 94^{o}C 30 s, 62^{o}C 30 s, and 72^{o}C 30 s, followed by a final extension step of 5 min at 72^{o}C.

dCAPS Analysis

A dCAPS marker for the 2078 mutation (Asp to Gly) was developed in this research to facilitate rapid and accurate identification of mutant 2078-Gly alleles in resistant ryegrass populations. The primer pair ACCF1/EcoRV2078r (Table-2) amplifies a 353-bp fragment using the same PCR conditions. Following EcoRV digestion, individuals with homozygous-resistant 2078-Gly alleles would have an uncut band of 353 bp, while individuals with homozygous susceptible 2078-Asp alleles would have a digested band of 323 BP. Individuals with both susceptible and resistant alleles would have a combination of two resolvable bands.

Table-2. Primer for PCR reaction.

Primer	Sequence 5 -3
ACCF1	CACAGACCATGATGCAGCTC
EcoRV2078r	GCACTCAATGCGATCTGGATTTATCTTGAT

RESULTS AND DISCUSSION

Resistance to ACCase herbicides

Diclofop-methyl resistance was common in the ryegrass populations randomly collected from Firoozabad wheat fields. Of the 42 populations treated with diclofop-methyl, 60% displayed some level of resistance (Table-3).

Table-3. The number and percentage of resistant ryegrass populations in each category for each herbicide populations (TR) and the total number of populations tested (TP) are shown. Zero indicates fully susceptible populations, 1–19% survival results in classification as populations developing resistance, and >20% survival results in classification as resistant populations.

Category	Diclofop	Sethoxydim	Clodinafop	Mesosulfuron	Trifluralin	Pinoxaden
0	16(40)	15(62.5)	19(50)	42	36	42
1 -19	9(22.5)	6(25)	7(18.4)	0	0	0
> 20	15(37.5)	3(12.5)	12(31.5)	0	0	0
TR	24(60)	9(37.5)	19(49.5)	0	0	0
TP	40	24	38	42	36	42

As expected, the degree of resistance varied across populations, with 37.5% classified as resistant (>20% survival). A further 22.5% of populations were classified as developing resistance (1–19% survival) and 40% of populations were diclofop-methyl susceptible (Table-3). This indicates that diclofop-methyl resistance is now much more common in ryegrass populations across high herbicide use with single mode of action zone. This may be attributed to the different herbicide use and characteristics among agronomic zones which in turn may explain the different diclofop- methyl selection pressure. Regions with greater diclofop-methyl usage may experience a higher frequency of resistant populations. This pattern was also found in the survey of Owen *et al*., 2007. For example, region C has less herbicide usage which is reflected by the lowest degree of resistance. In this region, 19% of the ryegrass populations were resistant ryegrass compared with high herbicide use areas (e.g. A, B) where 56% and 45% of the ryegrass populations had resistant plants respectively (Table-4).

Testing for Target Site Resistance With the Sethoxydim Herbicide

In general, resistance may be due to target site or non-target sites resistance as well as due to the occurrence of different ACCase mutations in ryegrass populations. (Tardif and Powles, 1994; Tardif *et al*., 1996; Zhang and Powles, 2006a; 2006b; Matthews *et al*., 1991; Holtum *et al*., 1992; Preston *et al*., 1996; Preston and Powles, 1998).

Table-4. The percentage of annual ryegrass populations that are resistant (R) or developing resistance (DR) to herbicides, and total resistance (TR) in each agronomic zone.

Zone	Diclofop			Sethoxydim			Clodinafop		
	DR	R	TR	DR	R	TR	DR	R	TR
A	17	39	56	8	23	31	8	36	44
B	15	30	45	12	11	23	14	24	38
C	10	9	19	5	7	12	5	9	14

Previous research has established that resistant ryegrass populations can not metabolise sethoxydim which may lead to sethoxydim resistance which indicates target site resistance (Tardif and Powles, 1994). Therefore, resistance to sethoxydim, can be endowed by mutations at the amino acid position 1781 (Delye *et al*., 2003; Menchari *et al.,* 2006; Zhang and Powles, 2006a). Although resistance to diclofop-methyl can be target or non-target site resistance. Our study revealed 24 diclofop-methyl resistant ryegrass populations. Of these populations, 37.5% were also found to be resistant to sethoxydim (Table-3). The majority of these populations originated from the A and B agronomic zones (Table-4). Thus, at least 37.5% of the ACCase herbicide resistant populations are likely to have target site resistance.

Resistance to Clodinafop-propargyl

Almost 50% of ryegrass populations collected from the Firoozabad wheat farms were found to exhibit resistance to the clodinafop-propargyl herbicide. Table-3 showed that 50% of ryegrass populations were resistant to clodinafop-propargyl (31.5% resistant and 18.4% developing resistance). In the A and B zones, 44 and 38% of populations were resistant to clodinafop-pro pargyl respectively (Table-4).

Substitution of Amino Acid Asp-2078-Gly and Ile-1781-Leu Endowing Resistance to Accase Herbicides in Ryegrass Populations

Ryegrass populations resistant to ACCase inhibiting herbicides are widespread in the Firoozabad site. Target site mutation Ile-1781-Leu was detected in the majority of the resistant individuals surveyed, ranging from 31% in A agronomic zone to 12% in C agronomic zone (Table-4). This study suggests that target site mutation within ACCase is common in populations of ryegrass. Mutation ranged from 37.5% in the target site to 62.5% in the non-target site. In this research only those ryegrass resistant (R) populations to diclofop (Table-3) were tested for mutation and we did not detect any point mutation 2078 – Gly in ryegrass populations through the molecular dCAPS approach (Figure 1).

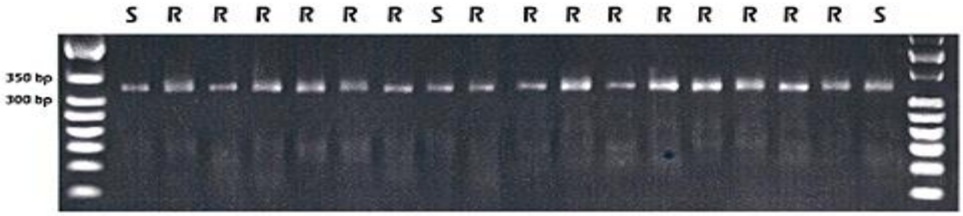

Figure1. DCAPS analysis individual *L.rigidum* Plants. The sizes of restriction enzyme 2078-Gly (EcoRV) digested fragments are 323bp.

CONCLUSION

During the last decades, more herbicides in controlling weeds in wheat fields of Iran had a same mode of action (ACCase-inhibiting herbicides) and this situation has caused emerging herbicide resistance in some weeds such as ryegrass. Continued selection of these populations with ACCase. - inhibiting herbicides will cause more mutations and resistance in ACCase group. The present study demonstrated that mesosulfuron+iodosufuron, trifluralin and pinoxaden herbicides may control most of the annual ryegrass populations. Both new herbicides (i.e. mesosulfuron+ iodosufuron and pinoxaden) are considered a low risk for resistance evolution and so will need to be used in rotation with other modes of action to delay the onset of resistance to the new herbicides. Resistance to diclofop-methyl and clodinafop-propargyl in annual ryegrass population is a serious problem for wheat producers across Firoozabad region. This study recommends management of ryegrass populations through herbicide rotation and adoption of integrated weed management strategies to prolong the efficacy of these herbicides.

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