INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0473/2017/19–6–1401–1408
DOI: 10.17957/IJAB/15.0437
http://www.fspublishers.org

Full Length Article

Resistance of Annual Ryegrass against Acetolactate Synthase-Inhibiting Herbicides in Wheat Fields: Field Evaluation and Molecular Analysis

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Abstract

Resistance to acetolactate synthase (ALS) inhibiting herbicides in annual ryegrass has evolved widely in Saudi Arabia but mutations conferring resistance have never been reported. In survey of 91 fields, representing seven ecological regions in Saudi Arabia four (57%) populations had resistance; whereas two (29%) populations had tendency of resistance development against ALS-inhibition herbicides. Use of ALS-inhibiting herbicides provided up to 73% control of ryegrass. Ten base pair substitution evolved in public sphere by the alignment of ALS cDNA sequence. Phenotypic alteration found in five mutants subsequently induced changes in amino acid. The Pro-197-Thr and Pro-197-Gln in Tabouk and Harad population, respectively present at earlier reported location with novel changes in amino acid threonine was unreported. Novel ALS-allele represented by Lys-98-Met mutants had methionine amino acid observed in whole Saudi population except resistant one with lysine. From Asp-185-Tyr novel allele was identified, aspartic acid in all Saudi population as well as in resistant allele and transformed into tyrosine. There exists resistance in ryegrass against ALS-inhibiting herbicides, which suggests use of alternate herbicides and/or development of integrated weed management strategies for wheat in Saudi Arabia.

Keywords: Annual ryegrass; Base pair substitution; Ecosystem; Herbicide resistance point mutation

Introduction

Annual ryegrass (*Lolium rigidum* Gaudin) is noxious weeds affecting wheat production globally. Chemical herbicides are mostly used by farmers from last many years to control weeds because these are easily available have high efficacy and are less expensive. Conversely, excessive herbicides usage causes herbicide resistance along with environment pollution (Farooq *et al.*, 2011; Heap, 2017).

Herbicides targeting acetolactate synthase (ALS) enzyme are commonly used worldwide. Inhibition of ALS leads to starvation of plant to amino acids, isoleucine, valine and leucine (Tranel and Wright, 2002), and causes decreases biosynthesis of proteins leading to rapid cessation of growth. Nonetheless, ALS resistance (group B) is one of the most commonly evolved resistances (Beckie, 2006). First case of ALS inhibitor-resistance in annual ryegrass was reported in Australia during 1986 (Heap and Knight, 1986) followed by target specific resistance in prickly lettuce (*Lactuca serriola* L.) in 1987, in the USA (Mallory-Smith *et al.*, 1990) and by 2015, 158 species of ALS-resistant weeds in 32 countries were reported (Heap, 2017).

ALS-resistant ryegrass populations are comprised of either mixed individuals with resistance endowing mutations or heterozygous/homozygous individuals for various combinations (one or two) of varied mutation (Yu *et al.,* 2007). Herbicide resistance may involve a single mutation or functional modifications. Herbicides resistance with specific target site in various weeds against multiple herbicides has been observed. For instance, Retzinger and Mallory-Smith (1977) noted ryegrass plants in Australia with multiple resistances to several herbicides such as cyclohexanedione, sulfonylurea, dinitroaniline, triazine and substituted urea.

Derived cleaved amplified polymorphic (dCAPS) markers has been used to identify resistant ALS-mutations among the four (resistant) population. These markers are quite helpful in the identification of individual (homozygous or heterozygous) endowing mutation for specific resistance and for the assessment of resistance level to ALS herbicides from population (Yu *et al.,* 2008). For instance, several scientists (Yu *et al.*, 2008; Delye *et al.*, 2009) reported that, mutations at the same two resistance ALS codon positions from eight established in other grasses and broad leaved

To cite this paper: Al-Doss, A.A., S.A. Al-Faifi, A.I. Ghazy, A.A. Salem, M.A. Khan, M. Althamrah, M.H. Ammar, M. Farooq and H.M. Migdadi, 2017. Resistance of annual ryegrass against acetolactate synthase-inhibiting herbicides in wheat fields: field evaluation and molecular analysis. *Int. J. Agric. Biol*., 19: 1401–1408

species have been found in ryegrass. In ryegrass, resistance conferred by mutations at Pro-197 to sulfonylureas, however, mutation at Trp-574 confers resistance to sulfonylureas and imidazolinones herbicides. The ALS mutations have been reported in numerous weeds species (50; monocotyledonous and dicotyledonous) including ryegrass (Ta *et al.,* 2007; Delye *et al.,* 2009).

Increasing annual ryegrass infestation and difficulty to control is a severe threat to wheat production (Justice *et al.,* 1994; Ablerto, 2001). Like other parts of the world, chemical control of annual ryegrass in wheat fields of Saudi Arabia is not very effective now as it was before. For instance, application of diclofop-methyl to infested wheat field didn't suppress ryegrass populations (Al Mutlaq, 2002).

Information on type and distribution of annual ryegrass populations with resistance against ALS-inhibiting herbicides in Saudi Arabia is limited. In a recent study, we found that some plants of annual ryegrass survived even at the higher application rate of ALS-inhibiting herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr in Tabouk and Qassim (Al-Doss *et al.,* 2013). Nonetheless, magnitude or degree of resistance of annual ryegrass to ALS-inhibiting herbicides has not studied earlier. Moreover, responses of annual ryegrass to ALS-inhibition herbicides, other than mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr, under its different density levels in wheat field have not been evaluated. Furthermore, studies on type and distribution of ALS-mutation, inhibition and molecular basis in annual ryegrass populations from Saudi Arabia are lacking.

This study was therefore, conducted to highlight the resistance of annual ryegrass against herbicides; to observe ALS-inhibition resistance and to evaluate the influence of two commercial ALS-inhibiting herbicides on weed survival and wheat performance in Saudi Arabia. Moreover, potential mutations conferring ALS-herbicides resistance in population of annual ryegrass damaging wheat in Saudi Arabia were also monitored.

Materials and Methods

Field Survey and Sample Collection

A comprehensive field survey for annual ryegrass populations was conducted during the wheat growing season 2010. Field survey covered 91 fields with history of annual ryegrass infestation within seven regions (Wadi El-dawaser, Tabouk, Qassim, Hail, Aljouf, Harad and Aldawadmi) (Fig. 1). Five fields with history of ryegrass infestation and ALS herbicide use within each region (total of 35 fields) were selected for sampling. Seeds of annual ryegrass were collected from 50 annual ryegrass plants in each field at maturity stage. Sensitive population was kindly provided by Syngenta AG, Riyadh, Saudi Arabia.

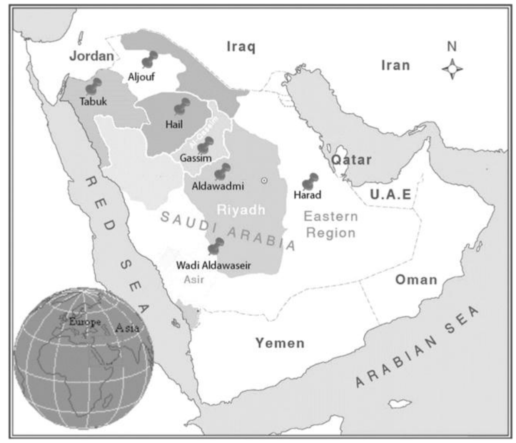

Figure 1: Locations of field survey of annual ryegrass populations in Saudi Arabia

Evaluation of ALS Inhibiting Herbicides

The experiment was conducted at an isolated area at Dirab Research and Experimental Station (24º 43' 34" N and 46º 37' 15" E), College of Food and Agricultural Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia for two consecutive years. Soil at experimental site was sandy clay loam with pH 8.15 and electrical conductivity 2.1 dS m^{-1}. The influence of two most widely used ALS-inhibiting herbicides in Saudi Arabia, iodosulfuron-methyl sodium (hussar) and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr (atlantis), on grain yield of spring wheat at three seeding densities of annual ryegrass infestation (100, 200 and 300 seed m^{-2}) was evaluated using a mixed population of annual ryegrass.

Seeds of wheat cultivar Yocora rojo were sown on November 27, 2010 and 2011 at a seed rate of 160 kg ha^{-1} in 6 m^2 experimental plots (0.5 m spaced 3 m long 4 rows). Irrigation application was done when quantity of water (evaporated) from "class A pan" reached at 45 mm and nitrogen and phosphorus were applied as recommended, NP (225 and 175 kg ha^{-1}) using sources calcium super phosphate and ammonium sulphate, respectively. Phosphorus full dose was basal applied, whereas one third amount of nitrogen were also basal applied, however left dose at tillering and leaf boot stage in two equal splits.

The ALS-inhibiting herbicides iodosulfuron-methyl sodium 100 g L^{-1} (hussar) and mesosulfuron-methyl 10 g kg^{-1} + iodosulfuron-methyl-sodium 2 g kg^{-1} + mefenpyr 30 g kg^{-1} (atlantis) were applied four weeks after planting at 3-4 leaf stage of annual ryegrass. The experiment was arranged in randomized complete block design with factorial arrangement with four replications.

Annual ryegrass density prior to herbicide application and three weeks after application was recorded to calculate survival percentage. Seedling dry weight of annual ryegrass was also taken. At harvest, biological and grain yields of wheat were recorded for all treatments. Data taken for both study seasons, were pooled and were subjected to analysis of variance using statistical software SAS (SAS, 2004).

Field Evaluation of the Annual Ryegrass Populations

This experiment was also conducted at an isolated area at Dirab Research and Experimental Station (24º 43' 34" N and 46º 37' 15" E), College of Food and Agricultural Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia. Experimental soil was sandy clay loam having pH 8.15 and electrical conductivity 2.1 dS m^{-1}. The collected annual ryegrass populations and the sensitive populations were planted on December 15, 2012 and 2013 at 200 seeds m^{-2}. The experiment was laid out in randomized complete block design with factorial arrangement having four replicates. Three weeks after planting, mesosulfuron-methyl 10 g kg^{-1} + iodosulfuron-methyl-sodium 2 g kg^{-1} + mefenpyr 30 g kg^{-1} (atlantis) was applied at 350 mL ha^{-1}.

Annual ryegrass density was noted prior to herbicide application and three weeks after application and the survival percentage was determined. At maturity, plant height, flag leaf area, number of tillers m^{-2}, days to flowering, spike length and annual ryegrass biological yield was recorded. Effect of herbicides was determined by recording the mortality rate of seedling after three weeks of herbicide application. If≥20% of individual from population persist after herbicide treatment that were rank as resistant; whereas populations with 1–19% survival were classified as evolving resistance (Owen *et al.,* 2007) and the populations with less than 1% survival as sensitive. Data recorded for both experimental seasons were pooled and were subjected to analysis of variance using statistical software SAS (SAS 2004).

DNA Extraction

Eight populations of annual ryegrass were germinated and grown in a growth chamber at college of Food and Agricultural Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia in pots filled with peat-moss and sand (1:2) and maintain for 15 days. Leaves were collected and dipped in (liquid) nitrogen nitrogen. DNA was extracted from 8 populations as single plant. Fifty DNA samples from each population were isolated using EZNA plant DNA isolation Kit (OMEGA Bio-Tech, USA) following manufacturer's protocol. DNA was quantified using 0.8% agrose gel electrophoresis and spectrophotometery using Nanodrop2000 (Fisher Scientific, USA).

PCR and Sequencing

The extracted DNA was screened using six previously reported primers for detection of ALS resistant mutations (Table 1; Yu *et al*., 2008). Primary screening results showed monomorphic patterns across individuals within the same population. Hence, DNA samples from individual plants within each population were pooled and integrity confirmed on 1% agarose gel. Six positive fragments representing ALS- specific primers (alleles) were selected from positive PCR amplicons from resistant population Tabouk (based on greenhouse and field evaluation, data not shown) and cloned in pGEM vector (Promega, Madison, WI. USA) and sequenced using Applied Biosystems BigDye® Terminator v3.1 and v1.1 according to the manufacturer's protocols and analyzed in Applied Biosystems 3130*xl* Genetic Analyzer. Sequence analysis showed potential mutations in ALS region. To access sequencing reproducibility, confirm the existence of potential ALS resistance alleles among ryegrass populations of Saudi Arabia and to retrieve the full length gene a set of new six primers were developed based on sequences obtained (Table 1).

Polymerase chain reaction (PCR) was done in 25 µL volume containing 50 ng of genomic DNA, 12.5 µL of 2X *GoTaq* Green Master Mix (Promega) and 0.5 µM of each primer. The PCR was run with the following profile: 94°C for 4 min, 35 cycles of 94°C for 30 sec, X°C for 30 sec and 72°C for 30–120 sec followed by a final extension step of 5 min at 72°C. X refers to the respective annealing temperature used for each primer pair (Table 1). PCR was carried out to confirm reproducibility. Cleaned elution of the amplified PCR of ALS fragments were used for TA cloning using pGEM Easy cloning kit (Promega, Madison, WI.USA) flowing manufacture's recommended protocol.

Transformed bacterial colonies were grown on agar LB solid medium supplemented with carbenicillin (100 µg mL^{-1} final concentration) as a selectable marker. Positive bacterial colonies were cultured on LB liquid medium overnight and minipreped using the QIAprep spin MiniPrep Kit (QIAGEN, Hilden, Germany). Sequencing PCR reactions were performed in a thermal cycler by using Applied Biosystems BigDye® Terminator according to manufacturer protocols. PCR products were analyzed in Applied Biosystems 3130*xl* Genetic Analyzer. A total of 384 picked positive clones were sequenced. High quality sequences were used to assemble contigs using Codon Code Aligner software (CodonCode Corporatio, Dedham, MA, USA). Sequences were viewed; vector trimmed and edited using CodonCode aligner. Sequences row data were analyzed, their quality were assessed using Phred score. Through individual submission, sequence obtained were deposited to NCBI (gene bank). A total of 18 assembled contigs were deposited (KF690243 - KF690257, KM000067, KM000068 and KJ953895), (http://www.ncbi.nlm.nih.gov/nuccore/).

Results

Field Survey

Severity of infestation varied within and among regions. Even within a field, the service roads, the first round and the outside border were the most weed infested areas. The use

Table 1: Primers used to detect potential ALS resistant alleles in studied ryegrass populations

Primer pair	Sequence (5-3)	Annealing temp (°C)	Product size (bp)	Reference
ALSF 122	GGGCGCCGACATCCTCGTCG	57	491	Yu *et al.* (2008)
ALSR197	ATCTGCTGCTGGATGTCCTT			
ALSF197	ACTCCATCCCCATGGTGGC	55	232	Yu *et al.* (2008)
ALSR197	ATCTGCTGCTGGATGTCCTT			
ALSF376	ATTCTCTATGTTGGCGGTGG	55	440	Yu *et al.* (2008)
ALSR376	CTTTTCTGCTCCAACTC			
ALSF197	ACTCCATCCCCATGGTGGC	59	1396	Yu *et al.* (2008)
ALSR574	ATAGGCAGCATGCTCCTG			
ALSF574	TGGGCGGCTCAGTATTACAC	55	532	Yu *et al.* (2008)
ALSR653	TCCTGCCATCACCTTCCATG			
ALSF574	TGGGCGGCTCAGTATTACAC	55	479	Yu *et al.* (2008)
ALSR574	ATAGGCAGCACATGCTCCTG			
ALSFL	GCAACTGCCACTTCGACAGC	59	1848	Sequence based developed primers in this study
ALSRL	GCAGCACATGCTCCTGGTG			
ALSFC-A	CTCTGCTAACTGGGAGCAAAGCA	60	742	
ALSRL	GCAGCACATGCTCCTGGTG			
ALSFD-H	TCTGCTAACTGGGAGCAAAGCAC	60	793	
ALSRL	GCAGCACATGCTCCTGGTG			
ALSFL	GCAACTGCCACTTCGACAGC	61	500	
ALSR-1	GCGGAGACGAGGTTGGTGG			
ALSFS2	CTCTATGTTGGCGGTGGCTGC	59	500	
ALSRS2	CCTGGATAGCATACTGCGGTGG			
ALSFS3	TGAGCTCACCAAAGGTGAGGC	59	450	
ALSRL	GCAGCACATGCTCCTGGTG			

of herbicide was common in all regions. Ten different herbicides were identified as chemical control of annual ryegrass populations. Different ALS-inhibiting herbicides being used in the region to control annual ryegrass include: triasulfuron, iodosulfuron-methyl sodium and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr. However, iodosulfuron-methyl sodium and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr were the most common ALS-inhibiting herbicides used. Generally, farmers tended to use one herbicide but in heavily infested fields, some farmers follow the agriculture extension recommendation of using two different herbicides for better control of annual ryegrass populations such as Prosulfocarb as pre-emergence and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr as post-emergence herbicide.

Evaluation of ALS Inhibiting Herbicides

Field evaluation of two ALS-inhibiting herbicides (iodosulfuron-methyl sodium and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr) had significant effect on survival and seedling dry weight of annual ryegrass weed, biological and grain yields of wheat (Table 2). Maximum survival of annual ryegrass was noted, where 100 g m^{-2} seeds were sown in control treatments, which was followed by 200 and 300 g m^{-2} seeds in the same treatment (Table 3). Although both ALS-inhibiting herbicides suppressed the survival of annual ryegrass; however, mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr was more effective at all seeding densities of annual ryegrass (Table 3). In this regard, minimum survival of annual ryegrass was noted from the application of mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr where 300 g m^{-2} seeds were planted (Table 3). Seedling dry weight of annual ryegrass tended to increase with increase in its seeding density irrespective of herbicide used. However, herbicide application substantially decreased the seedling dry weight of annual ryegrass. Maximum seedling dry weight of annual ryegrass was noted where 300 g m^{-2} seeds were planted from control treatment; whereas minimum seedling dry weight of annual ryegrass was noted from application of iodosulfuron-methyl sodium with seeding density of 100 g m^{-2} seeds, which was followed by the application of mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr with the same seeding density (Table 3). Increase in seeding density of annual ryegrass caused substantial decrease in biological and grain yields of wheat from control and Hussar application. Maximum biological and grain yields of wheat were recorded from control treatment with no seed of annual ryegrass, which was followed by 100 g m^{-2} seeds of annual ryegrass from control treatment for biological yield (Table 3). However, grain yield was similar to application of mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr at

Table 2: Analysis of variance for field evaluation of two ALS herbicides

Source of variation	df	Ryegrass		Wheat	
		Survival	Dry weight	Biological	Grain
Herbicide treatments	2	**	**	**	**
Ryegrass seed density	3	**	**	**	**
Herbicide × Ryegrass	6	*	**	**	*

*, ** significant at p 0.05 and 0.01; df = degree of freedom

Table 3: Influence of ALS herbicides on survival and dry weight of ryegrass and yield of wheat under varying densities of ryegrass

Herbicide	Ryegrass seed density (m^{-2})	Ryegrass		Wheat	
		Survival (%)	Dry weight (g m^{-2})	Biological yield (g m^{-2})	Grain yield (g m^{-2})
Control	0	-	-	1612.0 a	561.1 a
	100	78.4 a	387.8 c	1460.2 b	511.5 ab
	200	78.1 ab	488.5 b	1318.5 bc	447.2 bc
	300	68.7 b	538.5 a	1265.1 d	426.7 c
Iodosulfuron-methyl sodium	0	-	-	1340.5 bc	536.5 a
	100	34.3 c	165.0 de	1175.3 e	519.0 ab
	200	30.4 cd	236.3 d	1102.8 e	496.3 ab
	300	25.8 cd	269.5 d	1058.4 ef	490.9 ab
Mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr	0	-	-	1367.5 bc	548.2 a
	100	31.1 cd	214.0 de	1177.1 e	530.0 ab
	200	28.8 cd	237.8 d	1015.6 ef	534.4 a
	300	23.6 d	266.0 d	1037.2 ef	545.1 a
LSD ($p \leq 005$)	-	9.4	62.6	125.3	43.3

Means sharing the same letter for a parameter don't differ significantly at $p \leq 0.05$

Table 4: Influence of herbicide application on performance of ryegrass pupations at various locations in Saudi Arabia

Population	Treatment	Pre-treat plant density (cm^{-2})	Post-treat plant density (cm^{-2})	Survival (%)	Plant height (cm)	Flag leaf area (cm^2)	Tillers (m^{-2})	Plant biomass (g m^{-2})	Days to flowering	Spike length (cm)
Sensitive	Control	165.33	165.33	100.00	81.20	13.91	1639.33	2782.00	-	-
	Herbicide	128.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-
Harad	Control	166.33	166.33	100.00	85.47	12.63	1479.00	2641.00	-	-
	Herbicide	161.33	0.00	0.00	0.00	0.00	0.00	0.00	-	-
Qaseem	Control	138.33	138.33	100.00	86.40	14.27	1183.67	2356.00	74.00	29.10
	Herbicide	148.33	47.00	31.50	54.27	8.13	693.67	925.67	76.00	24.56
Hail	Control	143.33	143.33	100.00	74.30	13.77	1489.67	2954.33	78.33	22.13
	Herbicide	137.33	42.00	30.97	54.27	8.73	776.00	1041.33	79.00	20.23
Aljouf	Control	139.67	139.67	100.00	72.17	10.27	1394.33	2905.33	79.00	24.33
	Herbicide	156.00	38.00	24.87	47.50	8.97	727.00	1068.33	79.00	24.10
W-Aldawaseir	Control	171.00	171.00	100.00	93.17	17.13	1546.67	2229.33	66.33	24.80
	Herbicide	170.00	13.33	7.87	52.97	10.53	286.67	440.00	77.00	23.43
Tabouk	Control	111.67	111.67	100.00	66.77	11.73	1450.67	2707.00	-	-
	Herbicide	98.33	50.33	53.40	27.40	6.00	725.00	682.33	-	-
Aldawadmi	Control	157.00	157.00	100.00	81.20	11.10	1274.33	2135.33	73.33	29.70
	Herbicide	150.33	6.00	4.10	40.87	5.83	110.67	98.57	79.00	21.60
Control (mean)		149.08	149.08	100.00	80.09	13.10	1432.21	2588.79	74.20	26.01
Herbicide (mean)		143.71	24.58	19.09	34.66	6.02	414.88	532.03	78.00	22.78
LSD at p 0.05										
Treatment		ns	11.36	3.30	18.55	1.02	559.20	ns	2.34	3.12
Population		23.90	ns	5.74	8.37	2.68	200.00	786.85	1.56	3.72
Treat × Pop interaction		ns						ns		ns

Pre-treat=Pre-treatment; Post-treat Post-treatment; W-Aldawaseir=Wadi Aldawaseir; ns=not significant at $p \leq 0.05$

seeding densities of 0, 200 and 300 g m^{-2} annual ryegrass seeds and application of iodosulfuron-methyl sodium with no seed of annual ryegrass (Table 3).

Field Evaluation of the Populations

The effect of herbicide treatment on the performance of vegetative growth of annual ryegrass plants was also pronounced. Plant height was significantly reduced in all populations (with mean of 57%). Maximum reduction in plant height was recorded in the Tabouk (59%) and Aldawadmi (50%) populations; however, minimum reduction (27%) was recorded in Hail population. The flag leaf area was also reduced by herbicide treatments. The overall reduction in flag area was 54%. Tabouk and Wadi El-dawaser were the most sensitive populations with reduction of 49 and 47%, respectively, whereas Aljouf was the least affected population (13%). Most pronounced effect of herbicide application on vegetative growth of annual ryegrass plants was recorded on number of branches/plant with the overall reduction of 71%. Two populations Aldawadmi (91%) and Wadi El-dawaser (79%) had the maximum reductions in this regard; whereas minimum reduction in number of branches (41%) was recorded in Qaseem population. Although no significant difference for plant biomass was recorded among herbicide treatment and control highly significant difference was recorded among the populations. Herbicide treatment delayed the days to flowering. The overall increase in days to flowering was 5%. Wadi El-dawaser (16%) and Aldawadmi (8%) were the most affected populations, while the other populations showed no effects. The spike length showed overall reduction among population reached (12%). The highest pronounced reduction was recorded for Aldawadmi (27%)

while the lowest (1%) for Aljouf population (Table 4).

Herbicide application provided more than 80% control. Maximum mortality was recorded in the sensitive and Harad populations (100%); whereas Aldawadmi and Wadi El-dawaser populations had 4.1 and 7.9% survival, respectively. Almost 86% of annual ryegrass collected from the seven populations had individuals exhibiting resistance to the ALS herbicide Atlantis. Four annual ryegrass populations (57%) exhibited resistance; whereas two populations (29%) were developing resistance (Table 5).

Sequences were analyzed and compared with known ALS-resistant gene from gene bank (ACN_AF310684). The sequence comparison confirmed the existence of several base pair substitutions among the different populations. Sequences were viewed and their quality was assessed using Phred score. The blastn and blastx were used for analyzing the sequences. Analysis and alignment of partial sequences with ALS-resistant reference gene showed evidence for potential new mutant(s) that may confer resistance of ryegrass populations to the herbicide Atlantis (Table 6). The obtained sequences were assembled in 18 contiges among the ryegrass populations successfully spanned entire gene region (Table 7).

The sequence analysis revealed ten potential mutations spanning the ALS-resistance gene (Table 6). Novel potential allele ALS was represented by Lys-98-Met mutant had methionine amino acid noticed in all population, otherwise the resistant one (Tabouk), where Lysine occurred. Similarly, from Asp-185-Tyr another allele was identified and aspartic acid was observed in resistant population and all Saudi (tested) population changed into Tyrosine. Therefore, from Tabouk (resistant population) only two mutants Asp-185-Tyr and Lys-98-Met were detected (Table 6). Whereas, Glu-105-Gly showed (ecotype-specific) mutant it represents, continuous and stable change in amino acid amongst overall tested population irrespective to their resistance level (Table 6). Nonetheless, all these mutants might be taken as (ecotype-specific) mutants as it provides differentiation among all tested and ryegrass population. Mutants such as I1e-462 and Glu-213–Lys, only occurred (once) in each E1-Dawaser and sensitive population, respectively (Table 6).

Table 5: Survival percentage of populations, classes and the total number and percentage of resistant, developing resistance and sensitive ryegrass populations

Population	Survival (%)	Class
Sensitive (introduced population)	0.00	Sensitive
Harad	0.00	Sensitive
Qaseem	31.50	Resistance
Hail	30.97	Resistance
Aljouf	24.87	Resistance
Wadi Aldawaseir	7.87	Developing resistance
Tabouk	53.40	Resistance
Aldawadmi	4.10	Developing resistance
Resistant populations	4/7= 57%	
Evolving resistance	2/7= 29%	
Sensitive population	1/7= 14%	

Zero indicates fully sensitive populations, 1–19% survival results in classification as populations developing resistance, and >20% survival

Discussion

Weed infestation is one of the major factors limiting crop productivity and account for about one third of total losses caused by all the pests (Chhokar *et al.*, 2012). In Saudi Arabia, annual ryegrass is considered as more problematic weed, posing threat to winter cereals particularly wheat fields in Saudi Arabia. Herbicides were quite effective in controlling this noxious weed. However, their excessive and repeated use has induced herbicide resistance in this weed.

In field, resistance level was more where more herbicides application was done indicating, in that cropping system resistance was already more frequent (Owen *et al.*, 2007; Loureiro *et al.*, 2010; Owen and Powles, 2010; Broster *et al.*, 2011, 2013; Owen *et al.*, 2011, 2012a, 2012b, 2014; Al-Doss *et al.*, 2013; Musunda et al., 2015).

The extent of the wheat grain yield reduction in this experiment was dependent on the annual ryegrass density. As weeds density increases, linear decrease in grain yield was observed (Table 3). Moreover, in addition to yield loss, the presence of annual ryegrass seed in wheat grain can lead to a substantial price dockage (Fast *et al.*, 2009). Although both ALS-inhibiting herbicides iodosulfuron-methyl sodium and mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr suppressed the survival of annual ryegrass; however, mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr was more effective at all seeding densities of annual ryegrass (Table 3).

In this study, the application of mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr (ALS inhibitor) at recommended rate caused 100% mortality of the sensitive and Harad populations (Tables 4, 5). However,~86% of annual ryegrass collected exhibited resistance (57% exhibited resistance and 29% evolving resistance) (Table 5). Nonetheless, the degree of resistance varied across populations (regions), which indicate that ALS resistance is now more common than sensitivity in annual ryegrass populations across the regions. Cropping intensities and crop rotations vary among the regions and therefore, different ALS herbicides put different selection pressure. Regions with intensive cropping systems like Tabouk, Qaseem, Hail and Aljouf have had more use of ALS-inhibiting herbicides and consequently there was a higher frequency of resistant populations. However, farmers in Wadi El-dawaser and Aldawadmi have more focus on vegetable and fruits production and less on wheat cropping-based crop rotations (less herbicide usage) and thus annual ryegrass in this region had the lowest degree of resistance. In these regions 7.87 and 4.10% of the populations had resistant annual ryegrass plants. This pattern was also found recently in Saudi Arabia (Al-Doss *et al*., 2013), where the

Table 6: Potential mutants detected across eight ryegrass populations

Mutated amino acids position /population	Mutant name	Reference arabidopsis	Reference resistance ryegrass	Sensitive	Harad	Qaseem	Hail	Aljouf	Aldawaseir	Tabouk	Dwadmi
28	Pro-28-Leu/Gln	Pro	Leu	Gln	Gln	Leu	Gln	Leu	Leu	Gln	Leu
35	Ser-35-Ala/Val	Ser	Ala	Ala	Ala	Ala	A	Val	Val	Ala	Val
37	Pro-37-Ala/Thr	Pro	Ala	Ala	Thr	Ala	Thr	Thr	Thr	Thr	Thr
98	Lys-98-Met	Lys	Lys	Met	Met	Met	Met	Met	Met	Lys	Met
105	Glu-105-Gly	Glu	Glu	Glu	Gly	Gly	Gly	Gly	Gly	Gly	Gly
185	Asp-185-Tyr	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Tyr	Asp
197	Pro-197-Gln/Thr	Pro	Q	Pro	Pro	Pro	Gln	Pro	Pro	Thr	Pro
213	Glu-213-Lys	Glu	Glu	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Glu
462	Ile-462-Thr	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Thr
490	Met-490-Ile	Met	Met	Met	Met	Ile	Ile	Met	Met	Met	Met

Table 7: The sequences and accession numbers of new mutants for ALS-herbicide resistance in Saudi Arabia submitted in Gene Bank

S. No	Name	Accession#	Type
1	KSU-ALS-LR-1	KF690243	Contig, Partial cDNA
2	KSU-ALS-LR-2	KF690244	Contig, Partial cDNA
s3	KSU-ALS-LR-3	KF690245	Contig, Partial cDNA
4	KSU-ALS-LR-4	KF690246	Contig, Partial cDNA
5	KSU-ALS-LR-5	KF690247	Contig, Partial cDNA
6	KSU-ALS-LR-6	KF690248	Contig, Partial cDNA
7	KSU-ALS-LR-7	KF690249	Contig, Partial cDNA
8	KSU-ALS-LR-8	KF690250	Contig, Partial cDNA
9	KSU-ALS-LR-9	KF690251	Contig, Partial cDNA
10	KSU-ALS-LR-10	KF690252	Contig, Partial cDNA
11	KSU-ALS-LR-11	KF690253	Contig, Partial cDNA
12	KSU-ALS-LR-12	KF690254	Contig, Partial cDNA
13	KSU-ALS-LR-13	KF690255	Contig, Partial cDNA
14	KSU-ALS-LR-14	KF690256	Contig, Partial cDNA
15	KSU-ALS-LR-15	KF690257	Contig, Partial cDNA
16	KSU-ALS-LR-C1	KM000067	Contig, Partial cDNA
17	KSU-ALS-LR-C2	KM000068	Contig, Partial cDNA
18	KSU-ALS-LR-C3	KJ953895	Contig, Partial cDNA

populations Tabouk and Qassim showed 33 and 30% of survival compared to sensitive and Harad populations (no survival) at the high rate of ALS-inhibiting herbicide Atlantis. Glasshouse studies conducted in two other regions of Saudi Arabia *viz.* Al-Kharj and Al-Qassim indicated that annual ryegrass from Al-Kharj was sensitive to herbicides Diclofop-methyl, Poast, Axiom and Maverick; whereas Al-Qassim selection was resistant to double field rate of diclofop-methyl (Al Mutlaq and Mallory-Smith, 2004).

Christopher *et al.* (1991) proposed two ALS-inhibitor resistant mechanisms in weeds; one is target-site resistance where ALS-inhibitor resistance is linked to an altered ALS enzyme and no longer sensitive to the herbicide. This resistance is inherited as a Mendelian dominant or partially dominant (Boutsalis *et al.*, 1999) a point mutation in the gene sequence that determines an amino acid change in the protein is causing ALS enzyme insensitivity to the herbicide (Tranel and Wright, 2002; Corbett and Tardif, 2006). The second however, herbicide detoxification that was observed in some biotypes of annual ryegrass and twitch grass (*Alopecurus myosuroides* Huds.) (Christopher *et al.*, 1991).

Eight ryegrass populations, of which 7 populations were collected from long term herbicides exposed wheat fields and one standard sensitive population were investigated in this study. The results proved the existence of some potential ALS-resistant mutants among ryegrass populations. One mutant occurred at same position of earlier ALS-reported mutants, while others represented new identified ones. Others showed ecotype specific mutants regardless the resistance level. In annual ryegrass, the molecular basis of ALS mutations has not been adequately studied. Only the Pro-197-Ser and the Trp-574-Leu mutations have been reported in ALS-resistant ryegrass populations (Delye *et al.*, 2009).

Among the ten identified mutants, two mutants occurred at position 197, Pro-197-Gln and Pro-197-Thr in Harad and Tabouk population, respectively. These two mutants occurred at same position reported earlier, yet with a novel amino acid change, Threonine in the resistant population Tabouk and Pro-197-Gln in the sensitive population Harad were not reported earlier. Other Lys-98-Met mutant offer (new) ALS-allele having methionine amino acid from all under observation population except those which have resistance such as (Tabouk), where Lysine present. Similarly, from Asp-185-Tyr new allele was identified, which have aspartic acid in (known) allele, which are resistant among all population except Tabouk which was resistant, where conversion into Tyrosine takes place (Table 6). The three mutants Pro-197-Thr, Lys-98-Met and Asp-185-Tyr are of an interest since they all occurred only in resistant population Tabouk (Table 6). Glu-105-Gly also occurred as ecotype (specific) mutant, where it highlights the constant change in amino acid from all tested population, irrespective to their resistance magnitude. Mutants which differentiate Saudi population from ryegrass population might be taken as ecotype-specific mutants. In Wadi-Dawaser and sensitive check, I1e-462-Thr and Glu-213-Lys occur only once, respectively. The remaining mutants represent dispersed directions, which have no specific trend/coherence with resistance *per se*. Mutations details and their respective accession numbers in gene bank are presented in Table 7.

PCR-based markers and sequence analysis used in this study revealed potential ALS herbicide resistant ryegrass genotypes within wild ryegrass populations growing in Saudi Arabia. These ALS resistant mutations are related to ALS inhibiting herbicide mutations (Yu *et al.,* 2008). This study established the pre-existence of a diversity of ALS gene mutations endowing resistance in ryegrass populations grown naturally in Saudi wheat fields. These findings could have an impact on herbicide management strategies applied.

Conclusion

Continuous and excessive use of herbicide having single mode of action eventually results weeds resistant to numerous herbicides and their evolution. Sensitivity of annual ryegrass biotypes to herbicides, their competitive ability and their adaptation mechanisms to environment should also be considered for development of various strategies for the control of resistant biotypes. Herbicide Atlantis was more effective for controlling annual ryegrass in wheat. The results of this study confirm the occurrence of new mutations in ALS-resistant alleles in ryegrass populations. This problem may be overcome integrated weed management strategies.

Acknowledgements

This work was supported by NSTIP Strategic Technologies Program number (09-BIO681-02) in the Kingdom of Saudi Arabia.

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(Received 29 April 2017; Accepted 19 July 2017)