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SPECIAL TOPICS
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RESEARCH ARTICLE

Rigid Ryegrass (*Lolium rigidum* Gaud) Resistant to ACCase and ALS inhibitors in northeastern Iran

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Abstract: Background: Among the weeds in Iran, resistant *Lolium rigidum* Gaud is considered a troublesome weed in winter cereals due to its tendency to evolve cross (CR) and multiple resistance (MR) to herbicides.

Objective: This research examined the patterns and mechanisms of *L. rigidum* resistance to clodinafop-propargyl (CP) and mesosulfuron methyl+iodosulfuron methyl (MI).

Methods: Experiments were conducted on four putative-resistant *L. rigidum* biotypes and one susceptible biotype. The dose-response assay was performed on the biotypes with CP and MI. CR and MR were investigated with haloxyfop-R-methyl (HRM), sethoxydim (SD), pinoxaden (PN). and isoproturon+ diflufenican (ID) herbicides. An indirect study of the metabolism of herbicides was carried out using the cytochrome P450 monooxygenase (CYP450) inhibitors 1-aminobenzotriazole (ABT), piperonyl butoxide (PBO) and malathion. Finally, sequencing of ALS and ACCase genes was performed to investigate target-site resistance.

Results: All putative-resistant *L. rigidum* biotypes were resistant to CP, MI, and HRM, but susceptible to SD, PN, and ID. The indirect study showed that the P450 enzyme had no role in the evolution of resistance in *L. rigidum* biotypes. Resistance in this species was due to Ile-1781-Leu and Pro-197-Ser substitutions on ACCase and ALS encoding genes led to resistance, respectively.

Conclusions: Resistance in the studied *L. rigidum* biotypes to ALS and ACCase inhibiting was due of target site resistance. If these resistant biotypes are not controlled, they will become a problem for farmers in the region.

Keywords: Cytochrome P450, multiple resistance, TSR and NTSR, wheat

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1. Introduction

Due to its high nutritional value and its role in the country's food security, wheat is considered a strategic product in Iran, especially in the north of the country and Golestan province. Golestan province ranks second in the country, producing about 8% of Iran's wheat (Iran Agricultural Organization, 2021). Rigid ryegrass (*Lolium rigidum* Gaud) is a weed that has become resistant (R) to multiple herbicides (Yu, Powles, 2014a). This species is an important weed in Iran's cereal fields and is frequently found in agricultural fields in Golestan province, which has caused problems for wheat production in this region (Tavasoli et al., 2021).

Weeds are very efficient in reducing crop production (Zheng et al., 2011). Consecutive use of herbicide with a similar mode of action to control weeds is associated with increased herbicide resistance risk (Gherekhloo et al., 2016). In general, resistance mechanisms are divided into two categories: target site (TSR) and non-target site (NTSR) (Delye, 2013). NTSR is a change in a site other than the herbicide site of action and includes increased metabolism, reduced absorption and translocation, and herbicide detoxification (Hassanpour-bourkheili et al., 2021). In TSR, the herbicide binding site changes due to mutations in or overexpression of herbicide target genes (Gherekhloo et al., 2021). In TSR, changes in the TSR, including enzymatic and non-enzymatic proteins and cell division pathways, can result in resistance (Golmohammadzadeh et al., 2020). Derived cleaved amplified polymorphic sequence (dCAPS), allele-specific polymerase chain reaction (PCR), and sequencing are used to investigate molecular resistance in weeds (Gherekhloo et al., 2012; Hatami et al., 2016; Golmohammadzadeh et al., 2020; Hassanpour-bourkheili et al., 2021).

Currently, 50 species have evolved resistance to acetyl-CoA carboxylase (ACCase) inhibiting herbicides (Heap, 2022). ACCase inhibitors are categorized into aryloxyphenoxypropionates (APP1), aryloxyphenoxypropionates (APP2), cyclohexanediones (CHD) and phenylpyrazoline (PPZ) chemical classes (Hassanpour-bourkheili et al., 2021). ACCase inhibitors disrupt the activity of the ACCase enzyme in the plastid of monocots plants, while these herbicides do not affect the heteromeric ACCase enzyme present in dicotyledons (Hassanpour-bourkheili et al.

2021; Gherekhloo et al., 2021; Gherekhloo et al., 2020). One hundred seventy species (105 dicotyledonous and 65 monocotyledonous species) are reported to be resistant to acetolactate synthase (ALS) inhibitors (Heap, 2022). This enzyme is essential for the formation of branched-chain amino acids. Inhibitors of ALS include 5 groups of herbicides: sulfonylurea, imidazolinones, triazolopyrimidines, pyrimidinyl thiobenzoates, sulfonyl aminocarbonyl triazolinone (Yu, Powles, 2014a).

Resistance in *L. rigidum* has been reported to 14 herbicides from different chemical families (Heap, 2022). The farmers of Golestan province in the north of Iran reported lack of control of *L. rigidum* by ACCase and ALS inhibitor herbicides such as clodinafop-propargyl (CP) and mesosulfuronmethyl+iodosulfuron methyl (MI). Consequently, this research was conducted to understand the mechanism and pattern of resistance of *L. rigidum* to CP and MI herbicides.

2. Material and Methods

2.1 Plant material

Putative-resistant *L. rigidum* biotypes were collected from wheat fields of Golestan province in Iran in 2019. The susceptible (S) biotype seeds were collected from areas that had no history of herbicide applications. Information on the collected biotypes is presented in Table 1. The seeds were kept separately in bags and stored at 4 °C until the experiment was conducted.

Table 1 - The collection coordinates of the putative-resistant *L. rigidum* resistant biotypes from Golestan province, Iran

Biotypes	Coordinate point
R_1	(36° 53′ 45″ N 54° 23′ 20″ E)
R_2	(36° 47′ 40″ N 54° 05′ 44″ E)
R_3	(36° 53′ 28″ N 54° 04′ 57″ E)
R_4	(37° 22′ 36″ N 55° 23′ 37″ E)
S	(37° 32′ 59″ N 55° 27′ 43″ E)

2.2 Dose-response assay

In this experiment, first, to create uniformity in germination, the seeds were placed at 3 °C for 72 hours and in the next stage, they were transferred to an alternating temperature of 15 °C and 25 °C (12 hours at night and 12 hours during the day). Seedlings were sowed in pots with 10 × 10 × 10 cm dimensions. And then in 3 to 4 leaves, they were sprayed with the herbicides CP and MI with a calibrated knapsack sprayer with a flat fan nozzle (8003) at a pressure of 200 KPa. Each pot represented a replicate, and three pots were considered as the control without treatment. Sprayed herbicides and their rates are presented in Table 2. Twenty-eight days after spraying, sampling was done from the surface of the pots and dried in the oven at 75 °C for 72 hours. The results of the data related to the measurement of dry weight indicate the control percentage of biotypes.

2.3 Cross (CR) and multiple resistance (MR) assays

To investigate the CR and MR of *L. rigidum* biotypes with CP and MI herbicides, all biotypes were treated with haloxyfop-R-methyl (HRM), sethoxydim (SD), pinoxaden (PN) of ACCase inhibitors and isoproturon+ diflufenican (ID) of PSII inhibitors herbicides with rates applied is given in Table 3. The work steps were similar to the 2.2 section (dose-response assay), which lasted from October to December 2020. After the experiment was finished, by estimating the resistance factors, resistant biotypes have been classified by Beckie and Tardif's (2012) methods.

2.4 Herbicide metabolism assay

2.4.1 *Herbicide metabolism assay using ABT and PBO*

By metabolizing herbicides, the cytochrome monooxygenase P450 (CYP450) enzyme converts them into non-toxic secondary metabolites for the plant. Two chemicals ABT and PBO prevent the action of this enzyme. To determine the rate of herbicide metabolism, the Letouze and Gasquez (2003) method was used. All methods to create uniformity in germination are similar to section 2.2 (Dose-response assay). Then five seedlings per Petri dish containing filter paper were transferred, each Petri dish representing one replication. The

Table 2 - Herbicide treatment applied for dose-response assays

Herbicides	Manufacturer	Rate (g. ai. ha^{-1})	
		Biotype S	Biotype R
clodinafop propargyl (EC8%) (CP)	Ariashimi	0, 10, 20, 40, 80*, 160, 320, 640, 1280	0, 40, 80*, 160, 320, 640, 1280, 2560
mesosulfuron-methyl+iodosulfuron-methyl (OD 3%) (MI)	Bayer Crop Science	0, 2.25, 4.5, 9, 18*, 36, 72, 144	0, 9, 18*, 36, 72, 144, 288, 576

* recommended dose

experiment was carried out as a completely randomized design (CRD) and included four replicates. Experimental factors were: distilled water, CP at discriminating concentration, CP at discriminating concentration+ 10 mg. L^{-1} of ABT and CP at discriminating concentration+ 20 µl. L^{-1} of PBO. The discriminating concentration between S and R biotypes of CP herbicide was estimated before conducting this study (0.0196 mg ai. L^{-1}) (Tavasoli et al., 2021). After applying the above treatments, the petri dishes were incubated for 7 days at a temperature of 25 °C and after this period, the coleoptile length of the seedlings was measured and investigated based on the percentage compared to the control (Treatment with distilled water).

2.4.2 *Herbicide metabolism assay using Malathion*

Another method to investigate the effect of herbicide metabolism in the evolution of NTSR to ACCase and ALS inhibiting herbicides is the use of the insecticide malathion (MAL) as a CYP450 inhibitor that this enzyme causes the detoxification of these herbicides and finally the resistance of *L. rigidum* biotypes to these herbicides appears. This insecticide was used on the plant in combination with the herbicide CP and MI. The dosage of this insecticide along with herbicides that do not cause side effects to *L. rigidum* (complications other than the effect of inactivating CYP450) was 1,000 g. ai. ha^{-1} (Yu et al., 2009). To determine the effect of MAL on the metabolism of the ACCase and ALS inhibitors herbicides, the experiment was carried out as a CRD design and included three replicates with treatments including control (non-sprayed pots), and CP at 80 g. ai. ha^{-1}, MI at 18 g. ai. ha^{-1}, CP at 80 g. ai. ha^{-1}+ MAL at 1,000 g. ai. ha^{-1} and MI at 18 g. ai. ha^{-1}+ at MAL at 1,000 g. ai. ha^{-1}, the other steps included uniformity in germination and seedling formation of *L. rigidum* seeds and the method of spraying as in section 2.2. At the beginning of the treatment process, one hour before spraying with ACCase and ALS inhibitors herbicides, all biotypes were subjected to MAL at 0 or 1,000 g. ai. ha^{-1}. After four weeks, the biomass above the soil surface was harvested and transferred to a 75 °C oven for 72 hours to measure dry weight. To investigate the effect of CYP450 interference, resistance to herbicides was evaluated after treatment with MAL (an inhibitor of CYP450s) in resistant biotypes.

2.5 Identification of mutations in ACCase- and ALS Genes

2.5.1 *DNA extraction*

The extraction of DNA from R and S biotypes were carried out using the Speed tools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Spain) from 100 mg of fresh leaf tissues at the 3-4 leaf stage. DNA samples that had been quantitatively and qualitatively measured by Nanodrop 1000 were then used for PCR or kept in a freezer at a temperature of –20 °C until further use.

2.5.2 *ACCase and ALS Gene Sequencing*

Alopecurus myosuroides Huds ACCase (accession no. AJ310767) and ALS (accession no. AJ437300) genes were used for the numbering of amino acids. ACCase gene fragment containing the codon Ile-1781 was amplified using 5'-GATTGGCATAGCCGATGAAG-3' (F) and 5'-TGGACAACACCATTGGTAGC-3' (R) primers. Also, the fragment containing the codons Trp-1999 to Gly-2096 was amplified using 5'-AGCTTGGAGGAATCCCTGTT-3' (F) and 5'-GGGTCAAGCCTACCCATACA-3' (R) primers.

For the ALS encoding gene, the fragment containing the codon Ala-122 to Arg-377 was amplified using 5'-CCCATCCGAGCCCCGCAAG-3'(F) and 5'-ATCTAGCTCCTCATGCCACGAAC-3' (R). Also, the amplification of the ACCase fragment containing the codons Trp-574 to Gly-654 was done using 5'-GCACTGATTCGCATTGAGAACCTCC-3' (F) and the reverse primer 5'-AGAAATCCTGCCATCACCTTCC-3' (R). The sequencing experiment was carried out at the Center for Scientific and Technological Research of Extremadura (CICYTEX), Spain.

Table 3 - The rate of herbicides used for cross and multiple resistance assays related to *L. rigidum*

Herbicides	Manufacturer	Rate (g. ai. ha^{-1})	
		Biotype S	Biotype R
haloxyfop-R-methyl (EC 8%), (HRM)	Ariashimi	0, 13.5, 27, 54, 108*, 216, 432, 864	0, 54, 108*, 216, 432, 864, 1728, 3456
sethoxydim (EC 12.5%), (SD)	Arysta life science	0, 23.43, 46.87, 93.75, 187.5, 375*, 750, 1500	0, 187.5, 375*, 750, 1500, 3000, 6000, 12000
pinoxaden (EC 45%), (PD)	Syngenta	0, 4,21, 8.43, 16.87, 33.75, 67.5*, 135, 270, 540	0, 33.75, 67.5*, 135, 270, 540, 1080, 2160
isoproturon+ diflufenican(SC55%), (ID)	Agform Limited (UK)	0, 68.75, 137.5, 275, 550, 1100*, 2200, 8800	0, 550, 1100*, 2200, 4400, 8800, 17600, 35200

* recommended dose

2.6 Statistical analysis

The experiments were run twice. The interaction of herbicide doses and experimental run was not significant. Therefore, the data from the two repetitions were pooled.

Three-parameter log-logistic function (Equation 1) was fitted to the data related to dry weight using the using R software the (DRC package) (Ritz et al., 2015)

$$\text{Equation 1: } y = \frac{d}{(1 + (\exp\{b(\log(x) - \log(e))\}}$$

In the model, y is the shoot dry weight as a percentage of the control treatment; d is the coefficient corresponding to the upper limit of the response curve; b is the slope of the curve at point e; e is the effective concentration or dose to achieve 50% of the observed response; and x (independent variable) is the herbicide dose. The data from all dose-response tests have been analyzed separately. For all biotypes, the values of herbicide rate that causes a fifty percent reduction in plant growth compared to control (GR_{50}) were estimated. And then, for resistant biotypes, the $GR_{50}(R)/GR_{50}(S)$ ratio was estimated to evaluate the resistance factor (RF) (Cruz-Hipolito et al., 2012). The R software (R Core Team, 2020) (DRC package) was used to analyze the data related to dose-response assay (Ritz et al., 2015). The means were compared using the LSD method at $p<0.05$.

3. Results and Discussion

3.1 Dose-response assay using CP and MI

The results of this experiment after CP application showed that biotype R_3 had the highest GR_{50} with a value of 638.94 g. ai. ha^{-1}. The GR_{50} for the S biotype was estimated to be 20.76 g. ai. ha^{-1}. GR_{50} values for R_4, R_2, and R_1 biotypes were estimated to be 598.67, 541.65, and 530.92, respectively (Table 4). For MI, the R_2 biotype had the highest GR_{50} with a value of 866.09 g. ai. ha^{-1}, This value was estimated at 34.79 g. ai. ha^{-1} in S biotype. GR_{50} values for R_3, R_1 and R_4, biotypes were estimated to be 820.62, 808.76, and, 775.12 respectively (Table 4). The analysis showed that the GR_{50} values of R and S biotypes were different.

According to dose-response assay results, resistant biotypes had very high resistant factor (RF) values after the use of CP and MI herbicides (Table 4), which could be due to the continuous use of these herbicides and continued to create high selective pressure, on resistant biotypes of *L. rigidum* in wheat fields (Gherekhloo et al., 2016). In Iran, the most observed resistance is related to the family of ACCase-inhibiting herbicides such as CP, PN and Diclofop-methyl, which can be due to the consecutive use of these herbicides (Heap, 2022). In Greece, the population of *L. rigidum* showed resistance to MI (ALS inhibiting) and PN (Anthimidou et al., 2012).

3.2 Cross and multiple resistance

Dose-response assay results for CR and MR with the ACCase-inhibitors of HRM, SD and PN herbicides and ID of PSII-inhibitor confirm only the resistance of *L. rigidum* to only HRM (Table 5). The GR_{50} values for R_1, R_2, R_3, and R_4 biotypes were 264.76, 231.64, 273.11, and 214.61 g. ai. ha^{-1} respectively (Table 5), which were different compared to the GR_{50} for the S biotype, which was estimated at 28.03 g. ai. ha^{-1}. Also, RF values of *L. rigidum* biotypes resistant to HRM was estimated to be very high (Table 5).

Monoculture is still common in the region and crop rotation is rarely practiced. The farmers of the region used only one type of herbicide and did not diversify the herbicide mode of action. As a result, with this practice, the selection pressure to create resistance increased. Because ACCase and ALS inhibitors can control a wide range of weeds including *Avena sterilis*, *Phalaris* spp., *Bromus* spp., and *Hordeum* spp. in wheat, they are used abundantly in Golestan province, Iran. This will intensify the herbicide selection pressure on *L. rigidum* biotypes. Therefore, *L. rigidum* biotypes resistant to both ACCase and ALS inhibitors have emerged in wheat fields of this province.

Among the studied herbicides, ID, SD and PN were able to control resistant biotypes of *L. rigidum*. Therefore, these herbicides may be used to wipe out the resistant biotypes

Table 4 - Parameter estimates of *L. rigidum* response to clodinafop propargyl and mesosulfuron methyl + iodosulfuron methyl herbicides. Values in parentheses are standard errors

Parameter	clodinafop propargyl		mesosulfuron methyl + iodosulfuron methyl	
Biotype	GR_{50} (g. ai. ha^{-1})	RF	GR_{50} (g. ai. ha^{-1})	RF
R_1	530.92 (23.40)	25.55 (6.02)	808.76 (56.79)	23.25 (0.60)
R_2	541.65 (27.08)	26.08 (6.44)	866.09 (51.96)	24.88 (4.70)
R_3	638.94 (31.94)	30.77 (6.31)	820.62 (49.23)	23.58 (4.89)
R_4	598.67 (16.41)	28.81 (4.88)	775.12 (48.94)	22.28 (0.98)
S	20.76 (1.03)	----------	34.79 (2.08)	----------

GR_{50}= herbicide rate that causes a 50% reduction in plant growth compared to control; RF= which is the GR_{50} of the R biotype divided by the GR_{50} of the S biotype

Table 5 - Parameter estimates of *L. rigidum* response cross and multiple resistance assay. Values in parentheses represent standard errors

Herbicide	Biotype	GR_{50} (g. ai. ha^{-1})	RF	Herbicide	Biotype	GR_{50} (g. ai. ha^{-1})	RF
haloxyfop-R-methyl (APP)	R_1	264.76 (47.47)	9.44(1.81)	sethoxydim (CHD)	R_1	40.46 (7.52)	1.21 (0.38)
	R_2	231.64 (35.28)	8.269(1.76)		R_2	35.11 (5.73)	1. 05 (0.41)
	R_3	273.11 (38.02)	9.74 (1.80)		R_3	30.76 (5.84)	0.92 (0.31)
	R_4	214.61 (44.16)	7.65 (1.45)		R_4	39.45 (6.34)	1.18 (0.39)
	S	28.03 (1.85)	-		S	33.44 (4.34)	-
pinoxaden (PPZ)	R_1	11.48 (2.33)	1.10 (0.59)	isoproturon+ diflufenican (SC55%)(PSII)	R_1	100.91 (8.91)	1.02 (0.52)
	R_2	12.00 (2.01)	1.15 (0.53)		R_2	103.88 (8.35)	1.05 (0.55)
	R_3	11.27 (1.38)	1.08 (0.35)		R_3	94.50 (6.13)	0.96 (0.38)
	R_4	10.45 (2.06)	1.00 (1.06)		R_4	92.01 (5.45)	0.93 (0.35)
	S	10.44 (1.62)	-		S	98.94 (5.44)	-

GR_{50}: herbicide rate that causes a 50% reduction in plant growth compared to control; RF: which is the GR_{50} of the R biotype divided by the GR_{50} of the S biotype; APP: aryloxyphenoxypropionate; CHD: cyclohexanedione; PPZ: phenylpyrazoline; PSII: Photosystem II

Table 6 - Analysis of variance for herbicide metabolism assay

SOV	df	Mean Squares				
		R_1	R_2	R_3	R_4	S
Treatment	3	20.52 ns	25.55 ns	4.77 ns	27.66 ns	6864.30 **
Error	8	16.41	15.25	7.16	21.50	1.91
Total	14	---	---	---	---	---
CV (%)	---	4.21	4.08	2.72	4.85	4.90

Ns: non-significant; ** significant at p<0.01

and break the resistance cycle. However, other options such as product and herbicide rotation are necessary to lower the risk of herbicide resistance in the future. For example, because SD herbicide has been approved as a herbicide for canola fields, and since this herbicide has been able to control *L. rigidum* well, alternating canola with wheat and controlling *L. rigidum* in canola fields is a suitable solution to control this weed. According to the results of this study, *L. rigidum* showed a very low degree of resistance (0.93 to 1.21) against the use of ID from the PSII inhibitor family, so this herbicide is considered a suitable alternative to the ACCase and ALS inhibitor family. *A. sterilis* populations from Golestan Province in Iran have shown high resistance to HRM (Hassanpour-bourkheili et al., 2021). Some populations of *Rapistrum rugosum* and *Sinapis arvensis* L. from Golestan Province in Iran were resistant to several ALS-inhibiting (Hatami et al., 2016; Gherekhloo et al., 2018).

In the CR and MR studies of *L. rigidum* biotypes in Spain, resistance to diclofop methyl and clethodim from ACCase-inhibitor herbicides and MI and pyroxsulam+florasulam from ALS- inhibitor family were confirmed (Torra et al., 2021). In addition, in similar studies investigating resistance in other sites of herbicide action in Spain *L. rigidum* populations, resistance to chlortoluron from PSII inhibitors and prosulfocarb from fatty acid elongase inhibitors was confirmed (Torra et al., 2021). These findings indicate that *L. rigidum* has great flexibility to resist a wide range of herbicides. Kuk et al., (2008) also presented a similar report on the occurrence of CR and MR of diclofop-resistant *Lolium multiflorum* to ACCase and ALS inhibitors. According to these studies, CR and MR of *L. rigidum* populations to some ACCase and ALS inhibitors were confirmed.

3.3 Herbicide metabolism assay using ABT and PBO

The results show that the presence of herbicide along with ABT and PBO did not have any significant effect on the resistant biotypes of *L. rigidum* (Table 6), only seedling length of sensitive biotype when treated with herbicide and herbicide + ABT or PBO was found to be significantly lower than when treated with only distilled water (Table 6 and 7). Therefore, the present study showed that herbicide metabolism does not play a role in the development of resistance. Metabolic resistance to herbicides may cause CR and MR to other herbicides and as a result, their control becomes very difficult. To manage metabolic resistance, herbicides

should be used cautiously and at full rate and mixing and sequential use of herbicides should be avoided because they cause the induction of metabolic genes in the weeds (Yu, Powles, 2014a). Li et al. (2017) also investigated the effect of CYP450 inhibitors on the metabolism of fenoxaprop-P-ethyl herbicide on resistant *Beckmannia syzigachne* (Steud.) Fernald populations. During this study, pre-treatment of these populations with PBO with a maximum concentration of CYP450 inhibitor and then the application of fenoxaprop-P-ethyl decreased the GR_{50} values by 60.2%. As a result, this weed is controlled compared to the application of fenoxaprop-P-ethyl alone. Researchers can manage this resistance sooner by investigating metabolic resistance, such as *L. rigidum* to new selective herbicides (Yu, Powles, 2014a).

3.4 Herbicide metabolism assay using Malathion

According to the results of this experiment, the use of CP and MI herbicides alone and herbicides (CP, MI) + MAL (CYP450 inhibitor) did not have a noticeable and significant effect on the resistant biotypes of *L. rigidum* and only the sensitive biotype was affected with a significant difference. This treatment was given (Table 8). The application of herbicides (CP and MI) and herbicide + MAL inhibitor on the sensitive biotype compared to the application of distilled water alone caused a significant decrease in the dry weight (percentage of control) of this biotype and MAL did not affect herbicides efficacy for the *L. rigidum* (Table 9). The synergistic effect of MAL with bispyribac-sodium (Fischer et al., 2000) and penoxsulam (Yasuor et al., 2010) is also reported in ALS-resistant *Echinochloa phyllopogon* (Stapf.) Koss.

The results of the researchers' studies confirm the increase in the toxicity of ALS-inhibiting herbicides such as mesosulfuron-methyl (MSM) on resistant populations of *B. syzigachne* when it is pre-treated with MAL (2,000 g ai ha^{-1}) and the results indicate that the GR_{50} values of the resistant populations of this weed at the time of MSM application+ MAL showed a decrease of 65.6% compared to MSM use alone, and as a result, increases the toxicity of this herbicide (Li et al., 2017). The evolution of metabolic resistance occurs very quickly during the apply herbicide with the same site of action, especially cross-pollinating weed species because the genes responsible for this type of resistance are transferred quickly, which causes the selection and rapid multiplication of resistant populations. Different genetic diversity and high metabolic ability lead to the evolution of this type of resistance, which is considered a serious threat (Yu, Powles, 2014a).

3.5 Molecular study

3.5.1 *ACCase Gene Sequencing*

The results of the experiments confirm the substitution of Ile-1781 with Leu in all resistant biotypes of *L. rigidum* (R_1, R_2, R_3 and R_4) (Table 10). Changes in the amino acids of the carboxyl transferase (CT) domain is considered to be an obstacle to the binding of ACCase inhibitors in this domain and ultimately causes TSR resistance (Hassanpour-bourkheili et al. 2021; Gherekhloo et al., 2020). In general, researchers believe that the common cause of resistance to ACCase inhibitors such as APP, CHD and PPZ is the mutation at point 1781, which is considered the most frequent mutation in this domain (Golmohammadzadeh et al., 2020). So far, the substitution of three amino acids Leu, Thr, and Val, instead of Ile at position 1781 of the CT domain have been identified (Murphy, Tranel, 2019). The substitution of Ile instead of Leu was identified as the most common allelic variant at position 1781, which caused the pattern of extensive resistance in weeds such as *Lolium* spp. (Yu et al., 2007). The results of this study indicate that the mutation in Ile-178-Leu is the most important mutation in *L. rigidum* biotypes resistant to ACCase inhibitors in Golestan

Table 7 - Comparison of means for the susceptible biotype seedling length during herbicide metabolism assay. Similar letters indicate non-significant differences

Treatment	Dry weight (% of control)
control	100 a
clodinafop-propargyl	4.66 b
clodinafop-propargyl +ABT	4.00 b
clodinafop-propargyl +PBO	4.33 b

Table 8 - Analysis of variance for herbicide metabolism assay

SOV	df	Mean Squares				
		R_1	R_2	R_3	R_4	S
Treatment	4	9.69 ns	11.72 ns	7.56 ns	10.50 ns	6000**
Error	10	6.87	7.25	9.30	11.04	0
Total	14	---	---	---	---	---
CV (%)	---	2.70	2.77	3.13	3.42	0

Ns: * nonsignificant; ** significant at p<0.01

province, Iran, which is aligned with the findings of other researchers (Zand et al., 2009). The mutation at position 1781(Ile-1781-Leu) in resistant *L. rigidum* biotypes (R_1, R_2, R_3 and R_4), in addition to cross-resistance to CP, HRM may cause resistance to other families of ACCase inhibitors such as CHD and PPZ.

3.5.2 *ALS Gene Sequencing*

The results of molecular tests confirm the substitution of Pro-197- Ser amino acid in *L. rigidum* biotypes resistant to ALS inhibitor (R_1, R_2, R_3, R_4) compared to the susceptible biotype (S) (Table 11). This substitution in position 197 caused a high level of *L. rigidum* biotypes resistance to ALS inhibitors such as MI from the sulfonylurea (SU) family. Among the 12 amino acid substitutions in Pro197, the highest substitution rate is related to Pro197 to Ser197, which was reported in 21 weed species (Yu, Powles, 2014b). The researchers consider the substitution of Pro197 to Ser197 as the cause of cross-resistance in some dicot weeds to ALS inhibitors such as SU herbicides (Yu, Powles, 2014a). The replacement of amino acid Pro197 with Ser or Ala results in cross-resistance of *Sinapis alba* and *Conyza canadensis* resistant biotypes to herbicides such as SU, pyrimidinyl-thiobenzoate, and triazolopyrimidine (Zheng et al., 2011). Although mutations at different sites of the ALS gene produce a specific CR pattern (Powles, Holtum, 2017; Zheng et al., 2011), these mutational substitutions can result in different levels of resistance to a given herbicide (McCourt et al., 2005), and because creating a mutation at point Pro197 does not reduce fitness resistance spreads with high frequency (Stewart, 2009).

Table 9 - Comparison of means for the susceptible biotype dry weight herbicide metabolism assay. Similar letters indicate non-significant difference

Treatment	Dry weight (%of control)
control	100 a
clodinafop-propargyl	0 b
mesosulfuron methyl + iodosulfuron	0 b
clodinafop-propargyl + malathion	0 b
mesosulfuron methyl + iodosulfuron + malathion	0 b

4. Conclusions

The results show that after applying the ACCase inhibitior CP and HRM and the ALS inhibitor MI, all biotypes (R_1, R_2, R_3, R_4) showed high degrees of resistance. The sequencing results confirmed point mutations, Ile-1781-Leu in the *ACCase* gene and Pro-197-Ser in the *ALS* gene. No NTSR mechanism was detected in this study. Further investigation of resistance mechanisms can provide a better understanding of resistance phenomenon in this species.

Table 10 - Sequence alignment of the ACCase gene in the four *L. rigidum* (R_1, R_2, R_3, R_4) populations compared with the susceptible population (S). The arrow shows the mutation point

Biotype	↓Ile-1781													
S	G	V	N	I	H	G	S	A	A	I	A	S	A	Y
R_1	G	V	N	L	H	G	S	A	A	I	A	S	A	Y
R_2	G	V	N	L	H	G	S	A	A	I	A	S	A	Y
R_3	G	V	N	L	H	G	S	A	A	I	A	S	A	Y
R_4	G	V	N	L	H	G	S	A	A	I	A	S	A	Y

G: Glycine; V: Valine; N: Asparagine; I: Isoleucine; L: Leucine; H: Histidine; S: Serine; A: Alanine; Y: Tyrosine

Table 11 - Sequence alignment of the ALS gene in the four *L. rigidum* (R_1, R_2, R_3, R_4) populations compared with the susceptible population (S). The arrow shows the mutation point

Biotype	↓Pro197													
S	A	L	T	G	Q	V	P	R	R	M	I	G	T	D
R_1	A	L	T	G	Q	V	S	R	R	M	I	G	T	D
R_2	A	L	T	G	Q	V	S	R	R	M	I	G	T	D
R_3	A	L	T	G	Q	V	S	R	R	M	I	G	T	D
R_4	A	L	T	G	Q	V	S	R	R	M	I	G	T	D

A: Alanine; L: Leucine; T: Threonine; G: Glycine; Q: Glutamine; V: Valine; P: proline; S: Serine; R: Arginine; M: Methionine; I: Isoleucine; D: Asparatate

Author's contributions

AT: data curation, formal analysis, investigation, writing – original draft. JG and MO: methodology. JG: project administration, validation. JG and RP: supervision. JG, FG, EZ, AND RP: writing – review & editing.

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