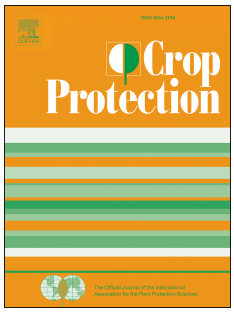

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Mechanism and pattern of resistance to some ACCase inhibitors in winter wild oat (*Avena sterilis* subsp. *ludoviciana* (Durieu) Gillet & Magne) biotypes collected within canola fields

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Abstract

Due to the reports regarding unsuccessful control of *Avena sterilis* subsp. *ludoviciana* (Durieu) Gillet & Magne by haloxyfop-R-methyl in canola fields, the following study was conducted to investigate the resistance of this weed to haloxyfop-R-methyl. Five out of 22 accessions were resistant to haloxyfop-R-methyl. These biotypes were then subjected to various rates of clodinafop propargyl, sethoxydim, pinoxaden and mesosulfuron methyl+ iodosulfuron-methyl herbicides and their and cross-resistance to clodinafop propargyl and sethoxydim was confirmed. However, no resistance was observed to pinoxaden and mesosulfuron-methyl + iodosulfuron-methyl herbicides. Indirect study of metabolism by P450 using 1-aminobenzotriazole and piperonyl butoxide showed that this enzyme had no contribution to occurrence of resistance in the studied biotypes. Allele-specific PCR results indicated that Ile-2041-Asn mutation is responsible for resistance of *A. sterilis* subsp. *ludoviciana* biotypes, which was confirmed by sequencing of the samples. Since pinoxaden negatively affects canola, the growers face a serious limitation in their choice for chemical management and thus, implementation of integrated weed management such as introduction of row crops such as faba bean in crop rotation and increasing the diversity of herbicide mode of action by cultivation of crops such as sugar beet in crop rotation may prove helpful. In fields under canola-wheat rotation, it is also possible to use pinoxaden in wheat. Also, trifluralin, cycloxydim and clethodim herbicides may be tested on *A. sterilis* subsp. *ludoviciana*. This was the first case of *A. sterilis* subsp. *ludoviciana* resistance to ACCase inhibitors in canola fields.

Keywords: ACCase inhibitors, Allele-specific PCR, Canola, Herbicide resistance.

Highlights:

- **Resistance to haloxyfop-R-methyl was detected within the biotypes**
- **Biotypes were cross-resistant to clodinafop-propargyl and sethoxydim herbicides**
- **Ile-2041-Asn mutation is responsible for occurrence of resistance in the biotypes**
- **No metabolic resistance was observed in the biotypes**
- **Biotypes were susceptible to pinoxaden and mesosulfuron methyl+ iodosulfuron-methyl**

1. Introduction

Weeds are a major threat to sustainable agriculture (Zhang et al., 2020). Over-reliance to herbicides for weed management and consecutive application of agrochemicals possessing similar mode of action led to emergence of a new threat which was termed as herbicide resistant weeds (Kudsk and Streibig, 2003). Resistance in weeds may be due to target site (TSR) or non-target site (NTSR) resistance (Délye et al., 2013) mechanisms. Non-target site resistance occurs when the alteration takes place at a site other than that of herbicide target, and may lead to reduced herbicide absorption and translocation, retention, enhanced herbicide metabolism and detoxification, increased herbicide sequestration in vacuoles and attenuated herbicide activity (Prather et al., 2000; Powles and Yu, 2010). Target site resistance involves mutations which alter the binding site of the herbicide. This type of resistance may also be evolved due to over-expression of herbicide target site gene (Heap, 2020). If a species possesses only one mechanism of resistance which enables it to survive herbicides from a subgroup within a specific herbicide group, the species is cross-resistant, whereas species with more than one resistance mechanism are classified as multiple resistant (Powles and Preston, 1995). Various studies are available on evolution of cross and multiple resistance in weeds (Gherekhloo et al., 2012; Keith et al., 2015; Li et al., 2017)

According to Heap (2020), 49 out of 262 resistant species, varieties and subspecies are associated with acetyl-CoA carboxylase (ACCase) inhibitors. aryloxyphenoxypropionates 1 (APP1), aryloxyphenoxypropionates 2 (APP2), cyclohexanediones (CHD) and phenylpyrazoline (PPZ) (Forouzesh et al., 2015). Pinoxaden herbicide is the only member of PPZ group, which along with the other ACCase inhibiting herbicides, targets homomeric ACCase enzyme in monocot plant plastids, whereas heteromeric ACCase enzyme found in dicots is not affected by these herbicides (Powles and Yu, 2010).

Certain mutations in ACCase encoding enzyme lead to development of TSR resistance in weeds. These mutations include eight unique mutation sites and 13 reported single nucleotide polymorphisms including Ile-1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Gly-2096-Ala (Délye, 2005), Ile-1781-Val, Asp-2078-Gly (Collavo et al., 2011), Trp-1999-Leu (Scarabel et al., 2011), Cys-2088-Arg (Yu et al., 2013), Trp-1999-Cys (Liu et al., 2007), Gly-2096-Ser (Beckie et al., 2012), Ile-2041-Thr (Guo et al., 2017) and Asn-2097-Asp (Cha et al., 2014). Various researchers have used molecular-based assays such as derived cleaved amplified polymorphic sequence (dCAPS) and allele-specific PCR in herbicide resistance confirmation studies (Gherekhloo et al., 2012; Dominguez-Valenzuela et al., 2017; Chen et al., 2018; Zhao et al., 2019).

High activity of enzymes including enzymes including cytochrome P450 monooxygenase (P450) and glutathione- S transferases (GST) families have also been linked with non-target site herbicide resistance (Letouzé and Gasquez, 2003). Cytochrome P450 monooxygenases are a rather large enzymatic family which are reported to metabolize various herbicides (Siminszky, 2006). Inhibitors such as 1-aminobenzotriazole (ABT) and Piperonyl butoxide (PBO) have the ability of detoxifying P450s and consequently, endow metabolic resistance to a species (Barta and Dutka, 1991; Hongchun et al., 2013).

Canola is one of the most important crops cultivated in the world and is also widely grown in Iran, especially in Golestan province located in the north of the country. Approximately 37% of canola production in Iran takes place in Golestan province (Kazemi et al., 2016). Farmers of this region usually sow canola or wheat as winter crops in rotation with a summer crop such as rice or soybean (Gherekhloo et al., 2016; Kamkar et al., 2014), and the majority of canola growers in Golestan province have adopted rain-fed production system (Soltani et al., 2014).

Weeds including wild oats (*Avena* spp.) can severely decrease the yield of canola (Lemerle et al., 2016) and may impose a yield loss of up to 32% to this crop (Bajwa et al., 2017). *Avena sterilis* subsp. *ludoviciana* (Durieu) Gillet & Magne is widely distributed in many temperate regions of the world, and may be found on all continents except Antarctica (CABI, 2016).

Many wheat and canola fields of the Golestan province are also heavily infested by *A. sterilis* subsp. *ludoviciana*, which has seriously damaged the canola production in this region (Hassanpour-bourkheili et al., 2017). Chemical management is one of the most common options in management of weeds plaguing this crop (Bodnar et al., 2019), so farmers of the region mostly use ACCase inhibitors such as diclofop

methyl, fenoxaprop-P ethyl and clodinafop propargyl to control *A. sterilis* subsp. *ludoviciana* in wheat and canola fields. However, *A. sterilis* subsp. *ludoviciana* has developed resistance to these herbicides due to their consecutive application. Thus, the only chemical option to control these APP-resistant *A. sterilis* subsp. *ludoviciana* biotypes in wheat fields of the region is the application of pinoxaden (PPZ) and acetolactate synthase (ALS) inhibiting herbicides. Also, canola growers mainly relied on haloxyfop-R-methyl after *A. sterilis* subsp. *ludoviciana* developed resistance to diclofop methyl, fenoxaprop-P ethyl and clodinafop propargyl (Gherekhloo et al., 2016).

There were reports on improper control of *A. sterilis* subsp. *ludoviciana* plants in haloxyfop-R-methyl treated canola fields of Golestan province, Iran.

Several researchers have reported the occurrence of *Avena* spp. biotypes resistant to ACCase inhibitors in wheat fields (Uludag et al., 2007; Cavan et al., 2008; Owen and Powles, 2016; Papapanagiotou et al., 2019), but no reports are available on resistance of *A. sterilis* subsp. *ludoviciana* to haloxyfop-R-methyl in canola fields. There were reports on failed control *A. sterilis* subsp. *ludoviciana* plants in haloxyfop-R-methyl treated canola fields of Kalaleh township, Golestan province, Iran. Thus, the following study was conducted to confirm and evaluate the resistance of *A. sterilis* subsp. *ludoviciana* to haloxyfop-R-methyl as well as identifying resistance pattern and molecular mechanism responsible for evolution of this resistance. Determination of the resistance pattern may greatly help to devise weed management strategies to control this weed.

2. Materials and methods

2.1. Plant material

The seeds of *A. sterilis* subsp. *ludoviciana* accessions were collected from 22 canola fields located in Kalaleh township located in Golestan province, Iran in June, 2015. The coordinates of these fields are presented in Table 1. These putatively resistant seeds had been collected from the fields which were under consecutive cultivation of canola for five years. The fields had been exposed to haloxyfop-R-methyl application for five consecutive years, and farmers were not satisfied with the efficacy of the herbicide. Susceptible biotype was collected from the locations in the same region with no history of chemical weed management. The fields from which the accessions were gathered are shown in Fig. 1. All further experiments were conducted from 2015 to 2017.

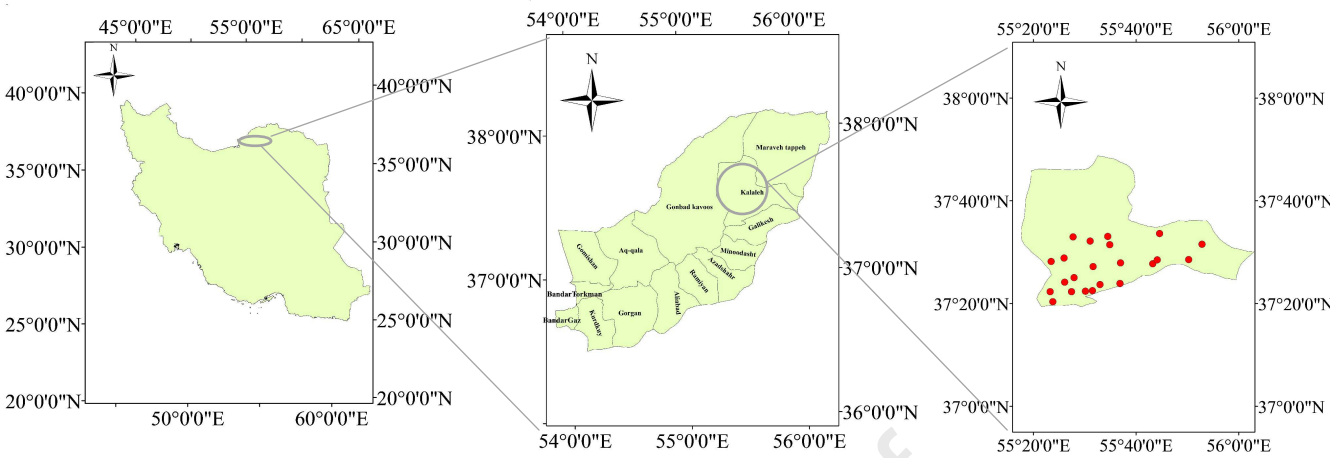

Fig. 1. Distribution map of the putative *A. sterilis* subsp. *ludoviciana* biotypes gathered from Kalaleh township, Golestan province, Iran.

Table 1. The coordinates of the putative *A. sterilis* subsp. *ludoviciana* biotypes gathered from Kalaleh township, Golestan province, Iran.

Biotype	Coordinates	Biotype	Coordinates	Biotype	Coordinates
RK-1	37° 27' 45" N, 55° 43' 15" E	RK-9	37° 28' 30" N, 55° 44' 07" E	RK-17	37° 25' 03" N, 55° 27' 55"' E
RK-2	37° 31' 28" N, 55° 34' 52" E	RK-10	37° 32' 10" N, 55° 31' 03" E	RK-18	37° 31' 33' N, 55° 52' 51" E
RK-3	37° 33' 38" N, 55° 44' 35" E	RK-11	37° 24' 09" N, 55° 26' 05" E	RK-19	37° 22' 24" N, 55° 30' 08" E
RK-4	37° 27' 55°" N, 55° 36' 58" E	RK-12	37° 22' 19" N, 55° 23' 15" E	RK-20	37° 23' 54" N, 55° 36' 52" E
RK-5	37°' 22' 19" N, 55° 27' 25" E	RK-13	37° 20' 22" N, 55° 23' 47" E	RK-21	37° 28' 11" N, 55° 23' 27" E
RK-6	37° 28' 33" N, 55° 50' 16" E	RK-14	37° 22' 31" N, 55° 31' 30" E	RK-22	37° 23' 42" N, 55° 32' 58" E
RK-7	37° 27' 12" N, 55° 31' 37°" E	RK-15	37° 28' 51" N, 55°' 28' 55°" E	S	37° 32' 59" N, 55° 27' 43" E
RK-8	37° 33' 05" N, 55° 34' 29" E	RK-16	37° 25' 13" N, 55° 27' 35" E		

2.2. Screening of the putative biotypes using haloxyfop-R-methyl

Screening of putatively resistant accessions was conducted from October to December in 2015 in the research greenhouse of Gorgan University of Agricultural Sciences and Natural Resources, Iran. The greenhouse temperature was regulated at 22/16 °C (day/night) with 12/12 h period of light/darkness and a relative humidity of 70%. The seeds were pre-chilled for 72 hours at 4°C to obtain uniform germination, and were then incubated at 25°C temperature for 24 hours (Tatari et al., 2018). Then, ten pre-germinated seeds of each biotype were sown in 25 cm diameter pots filled with 20 cm of silty-loam soil. The pots were arranged in a completely randomized design with three replications and each pot served as one replicate. Also, three unsprayed pots served as control for each biotype. Haloxyfop-R-methyl herbicide (under commercial name of Galant super, EC 10.8 % by Ariashimi, Iran) was applied at recommended rate (81 g a.i. ha^{-1}) at 3-4 leaf stage using a calibrated knapsack Matabi (Goziper Group, Spain) sprayer equipped with a flat fan nozzle (8003) at 200KPa. Each replication contained a control unsprayed pot so the data could be evaluated as percent relative to control. Four weeks after spraying, the number of survived plants

in each pot was recorded and calculated as percent relative to number of plants in control pot. Above-ground biomass in each pot was then cut and placed in an oven at 80° C for 48 hours and their dry weight was recorded and calculated as percent relative to dry weight of unsprayed treatment.

2.3. Dose-response assay using haloxyfop-R-methyl

Accessions which were able to preserve 50% survival and 80% dry weight compared to unsprayed control (Adkins et al., 1997) were determined as resistant biotypes and were selected for dose-response assay and evaluation of resistance factor. This experiment was carried out from January to March in 2016. Ten seeds were pre-germinated and sown in the pots as mentioned in screening section. The resistant biotypes were then sprayed with haloxyfop-R-methyl at 0 (control), 40.5, 81 (the recommended field rate), 162, 324, 648, 1296 and 2592 g a.i. ha^{-1} rates at 3-4 leaf stage using the sprayer mentioned in the 2.2 section . The herbicide rates used for susceptible biotypes were 0, 8.1, 16.2, 32.4, 48.6, 64.8, 81 and 162 g a.i. ha^{-1}. Four weeks after treatment, above-ground biomass in each pot was cut and dried in an oven at 80° C for 48 hours and their dry weight was recorded as percent relative to control.

2.4. Cross and multiple resistance assays

Dose response assays was conducted from October to December in 2016 as mentioned in the 2.3 section (dose-response assay) for clodinafop-propargyl (APP), sethyoxydim (CHD), pinoxaden (PPZ) and mesosulfuron-methyl + iodosulfuron-methyl(ALS) herbicides to investigate the cross and multiple resistance of haloxyfop-R-methyl-resistant *A. sterilis* subsp. *ludoviciana* biotypes. The herbicidal rates applied in cross and multiple resistance assays is presented in table 2. Other conditions and procedures were as same as described in the 2.3 section. The biotypes were then classified regarding their resistance factors according to Beckie and Tardiff (2012).

Table 2. The rate of herbicides used for cross and multiple resistance assays related to *A. sterilis* subsp. *ludoviciana*.

Herbicide	Applied rates (g a.i. ha^{-1})							
Clodinafop-propargyl (EC 8%)	0	40	80*	160	320	640	1280	2560
Sethyoxydim (OEC 12.5%)	0	187.5	375*	750	1500	3000	6000	12000
Pinoxaden (EC 45%)	0	33.75	67.5*	135	270	540	1080	2160
mesosulfuron-methyl + iodosulfuron-methyl (OD 3%)	0	9	18*	36	72	144	288	576

* Recommended field rate

2.5. Herbicide metabolism assay

The method described by Letouzé and Gasquez (2003) was used to determine the herbicide metabolism. The biotypes were first pre-germinated as described in the "screening" section. Then, five pre-germinated seeds from each biotype were placed in nine cm plastic Petri-dishes topped with Whatman paper No 1.

Each Petri-dish served as a replicate. The experiment was arranged as factorial based on completely randomized design with three replicates and the factors were cytochrome P 450 mono oxygenase inhibitors in three levels including distilled water, ABT at 10 mg/l and PBO at 20 µl/l and haloxyfop-R-methyl concentrations in two levels of distilled water and discriminating concentration. The haloxyfop-R-methyl concentration discriminating between the studied susceptible and resistant biotypes (based on EC_{80}) had been estimated previously (0.106 mg a.i. L-1, Hassanpour-bourkheili, 2019). The mentioned solutions were added to the Petri-dishes. Then, the Petri-dishes were kept in an incubator at 25 °C for seven days. The coleoptile length of the seeds was measured and expressed as a percentage of control (treated only with distilled water). This experiment was conducted from October to December in 2016.

2.6. Molecular assay

Young leaf tissues of haloxyfop-R-methyl-treated resistant biotypes as well as the susceptible biotype were sampled at 5-6 leaf stage from the main tiller of the survived plants in December of 2016. The samples were kept at -80° C until the beginning of the experiment. Doyle and Doyle (1990) protocol was used to isolate DNA of susceptible as well as resistant biotype (from three plants per biotype). Quantity and quality (A260/A280) of the extracted DNA samples were determined using a nano-spectrophotometer (Implen, Germany), which ranged from 1020 to 1155 ng L^{-1} and 1.8 to 1.9, respectively. Then, allele specific-PCR was performed in December of 2016 using primers presented in Table 3. A mix was prepared for each biotype which included 5 µL Taq DNA Polymerase 2x Master Mix Red (Ampliqon, Denmark), 0.5 µL for each forward and reverse primer, 0.3 µL $MgCl_2$ 1.5mM, 3 µL double distilled water and 1 µL DNA from each biotype. Then, the mixes were placed in a thermocycler (Lab cycler, Germany) for the chain reaction to begin. The program consisted of 3 minutes of initial denaturation at 95 °C followed by 35 cycles at 95 °C, 60 °C and 72 ° C all for 30 seconds. The final extension stage had a duration of 1 minute at 72 ° C. PCR products were then separated via electrophoresis in 2% agarose gel. The gel was dipped in SYBR Gold Nucleic Acid Gel Stain solution for 30 minutes and then exposed to UV radiation for analysis. As a confirmation for detection of mutation site by allele-specific marker, the biotypes underwent sequencing. After preparation of the mix using the same procedure as above, PCR was conducted using the primers shown in table 2. The program started with 4 minutes of initial denaturation at 94 °C followed by 35 cycles including 30 seconds at 90 °C, 30 seconds at 65/60 °C for 1781/1999-2096, respectively, and 1 minute at 72 °C. Final extension was performed for 10 minutes at 72 °C. Then, PCR products were sent

to Bioneer, South Korea for sequencing in January of 2016. The alignment was done using MultAlin software (Corpet, 1988).

Table 3. Primers used for PCR assays

Primer	Sequence	Annealing temperature (°C)	Assay
SOCT-α1F	AATACATGTGATCCTCGTGCAG	63	1999-2096 sequencing
SOCT- α 1R	TCCTCTGACCTGAACTTGATCTC	63	1999-2096 sequencing
SOCT-β1F	CATCATCTTTCTGTATGCCAGTGGG	65	1781 sequencing
SOCT- β 1R	CTGTATGCACCGTATGCCAAG	65	1781 sequencing
WT-F1	TTAGCTCTTCTGTTATAGCGCACA	Variable	Internal control
WT-R1	GAAGCTTGTTCAGGGCAGAA	Variable	Internal control
HR-Ft1	GATGGACTAGGTGTGGAGAACT	62	Detect Leu-1,781 allele
HR-RCvs1999	TTGGTAGCTGAATCTGGAAAA	62	Detect Cvs-1,999 allele
HR-RCvs2027	CCCACCAGAGAAGCCTCTA	64	Detect Cvs-2,027 allele
HR-RAsn2041	TTGATCCAGCCTGCAGAT	64	Detect Asn-2,041 allele
HR-RGlv2078	GCGATCTGGATTTATCTTGCTAC	66	Detect Glv-2,078 allele

Amino acid numbering was done according to *Avena myosuroides* chloroplastic ACCase (Genbank accession no. AJ310767).

2.7. Statistical analysis

All experiments were conducted twice. Screening, dose-response and metabolism assay experiments were repeated simultaneously, whereas the two experimental runs in the allele-specific PCR assay were repeated consecutively. The results of the two screening assay experiments were compared by performing an unpaired t-test. The results of all dose-response experiments (including cross and multiple resistance assays) were analyzed separately. Three- parameter log-logistic model (Equation 1) was used to fit the data associated with dry weight of plants expressed as a percentage of control (Ritz and Streibig, 2003):

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

in which y is shoot dry weight presented as percentage of control, d is upper limit, e is GR_{50}, i.e. the amount of herbicide required for 50% reduction in shoot dry weight and b is slope at GR_{50}.

Resistance factor (RF) was determined by dividing GR_{50} of the resistant biotypes to that of the susceptible one. A t-test was performed to find if there are any differences between the RF and 1 value.

Levene's test and Shapiro-Wilk statistics were used to test the regression analysis assumptions including homogeneity of variance and normality of residuals

After conducting Levene's test to assess the homogeneity of variances, combined analysis was used for indirect study of metabolism which consisted of two experiment repetitions.

Analysis of dose-response data, comparison of parameters among the biotypes via approximate t-tests and drawing of figures were done using the R software (R Core Team, 2020) (drc package) (Ritz et al., 2015). The R software was also used for the analysis of variance and conduction of the t-test ($p<0.05$) as well as

testing the regression analysis assumptions (Levene's test and Shapiro-Wilk statistics). Comparison of means was done using the least significant difference (LSD) method at $p<0.05$.

3. Results and discussion

3.1. Screening of the putative biotypes using haloxyfop-R-methyl

Since no differences were observed between the two experiment repetitions according to the t-test, the screening data were pooled. Five out of 22 *A. sterilis* subsp. *ludoviciana* accessions (27% of accessions) exposed to recommended field rate of haloxyfop-R-methyl were able to maintain their survival rate and dry weight higher than 50% and 80% compared to unsprayed control, respectively, and thus, were selected for dose-response assays. All these resistant biotypes had 80 to 100% survival rate, and their dry weights were not lower than 100% of control. The rest of the putative biotypes were classified as susceptible. The other putative biotypes which failed to prove resistant were discarded. Spraying errors including inability to spray the whole field, incorrect calibration of sprayer, using low-quality or expired herbicides or other factors may have been decisive in survival of these biotypes in the field. Susceptible biotype also did not survive the recommended dose (Table 4).

Table 4. Survival and dry weight of the collected *A. sterilis* subsp. *ludoviciana* biotypes after application of recommended haloxyfop-R-methyl dose based on the method described by Adkins et al. (1997). Values in parentheses represent standard error.

Biotype	Survival (% of control)	Dry weight (% of control)	Status	Biotype	Survival (% of control)	Dry weight (% of control)	Status
RK-1	20.00 (1.75)	35.65 (2.33)	S	RK-12	100.00 (0.00)	100.00 (0.00)	R
RK-2	35.00 (3.41)	42.20 (2.62)	S	RK-13	40.00 (2.66)	55.67 (1.75)	S
RK-3	30.00 (2.14)	33.33 (2.40)	S	RK-14	85.00 (4.36)	100.00 (3.58)	R
RK-4	40.00 (2.34)	60.66 (2.65)	S	RK-15	30.00 (2.46)	27.88 (2.32)	S
RK-5	100.00 (1.45)	100.00 (1.89)	R	RK-16	10.00 (3.62)	32.45 (3.24)	S
RK-6	10.00 (3.60)	25.00 (2.77)	S	RK-17	35.00 (2.47)	56.23 (3.68)	S
RK-7	0.00 (0.00)	0.00 (0.00)	S	RK-18	25.00 (3.85)	44.90 (3.46)	S
RK-8	100.00 (1.22)	100.00 (0.90)	R	RK-19	30.00 (2.55)	26.56 (3.13)	S
RK-9	20.00 (2.80)	45.12 (3.46)	S	RK-20	80.00 (4.95)	100.00 (3.67)	R
RK-10	30.00 (3.28)	38.90 (2.95)	S	RK-21	20.00 (2.75)	30.78 (3.60)	S
RK-11	25.00 (3.16)	23.78 (2.75)	S	RK-22	20.00 (3.20)	28.50 (3.45)	S
S	0.00 (0.00)	0.00 (0.00)	S				

S: Susceptible; R: Resistant

Screening for herbicide resistance may effectively lower the costs and eliminates the need for large spaces for dose-response experiments as well as being less time consuming compared to performing a dose-response assay for all putative biotypes (Beckie et al., 2000). Screening of *A. sterilis* subsp. *ludoviciana* biotypes collected from Greece showed that 89 percent of biotypes were resistant to diclofop methyl. Also, 71 and 61 percent of biotype showed resistance to fenoxaprop-P ethyl and clodinafop propargyl, respectively (Travlos et al., 2011). In another study on *A. sterilis* subsp. *ludoviciana* in Greece, 36 and 43 out of 125 biotypes were resistant to fenoxaprop-P ethyl and clodinafop propargyl herbicides, respectively

(Papapanagiotou et al., 2012). Populations of, *Avena fatua* L. gathered from Australia were screened herbicide resistance and it was observed that 47%, 17%, 2%, 23% and 2% of the population were resistant to diclofop methyl, fenoxaprop-P ethyl, clodinafop propargyl, sethoxydim and pinoxaden, respectively (Owen and Powles, 2009).

3.2. Dose-response assay using haloxyfop-R-methyl

Haloxyfop-R-methyl dose-response assay (Fig. 2) showed that no differences were observed among the resistant biotypes regarding the estimated GR50 and RFs in the first and second experiments. There was a significant difference between the GR50 of the susceptible and resistant biotypes. All resistant biotypes had statistically similar GR50 and RFs, and there was a significant difference between the RFs and the value 1. (Table 5).

The highest GR_{50} value was estimated 268.41 g a.i.ha^{-1} for RK12 biotype, and RK8, RK5, RK14, RK20 and S biotypes were respectively in next place with GR_{50} values of 260.60, 253.44, 222.33, 205.53 and 14.47 g a.i.ha^{-1}.Susceptible biotype had the highest slope at GR_{50} point (1.59) which suggests greater decline in growth reduction as 1 gram per hectare increase in active ingredient of haloxyfop-R-methyl compared to other biotypes, which had slightly lower b values. In other words, susceptible biotype had a higher declivity rate. All biotypes had significantly high resistance factors, ranging from 14.19 to 18.54 (Table 5) (Fig. 2).

According to the results, biotypes had high resistance factors to haloxyfop-R-methyl. This indicates that the growers imposed an intense selection pressure on *A. sterilis* subsp. *ludoviciana* biotypes in canola fields of the region through continuous application of haloxyfop-R-methyl. *Avena sterilis* subsp. *ludoviciana* populations from South Australia exhibited high levels of resistance to haloxyfop (Mansooji et al., 1992). Seefeldt et al. (1994) investigated the resistance of *A. fatua* biotypes from Oregon, USA to haloxyfop herbicide. They found out that the resistance factor of the resistant biotypes were 7.6 and 5.8.

b a

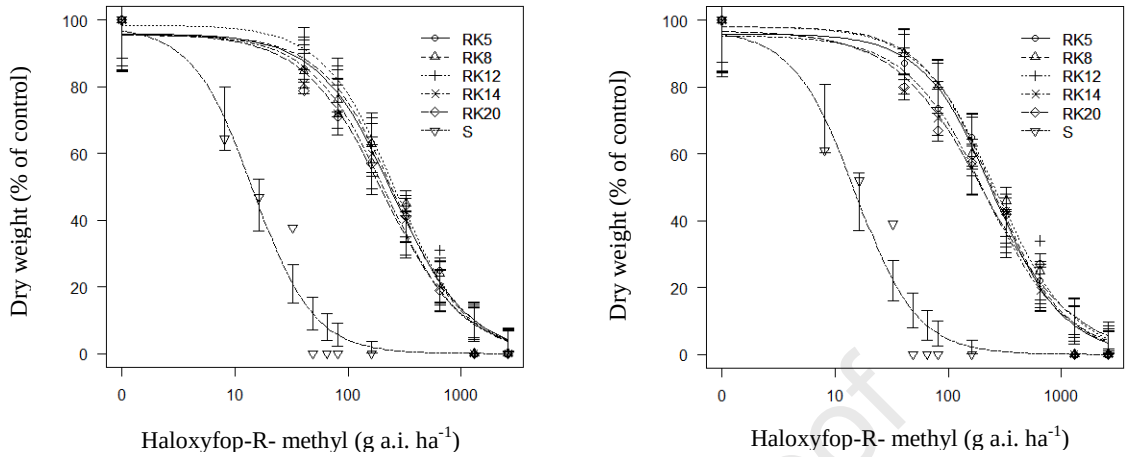

Fig. 2. Dose-response of *A. sterilis* subsp. *ludoviciana* biotypes to haloxyfop-R-methyl. a) first experiment; b) second experiment. The estimated GR_{50} and RF were presented in Table 5

Table 5. Parameter estimates of *A. sterilis* subsp. *ludoviciana* response to haloxyfop-R-methyl herbicide. Values in parentheses represent standard error.

Parameter / Biotype	First experiment		Second experiment	
	GR_{50} (g a.i ha^{-1})	RF	GR_{50} (g a.i ha^{-1})	RF
RK5	251.80 (43.93)^{a}	16.70 (4.95)^{a}**	249.55 (41.37)^{a}	17.29 (4.81)^{a}**
RK8	252.73 (41.33)^{a}	16.76 (4.84)^{a}**	257.09 (40.45)^{a}	17.81 (4.72)^{a}**
RK12	264.86 (45.46)^{a}	17.56 (5.15)^{a}**	261.71 (39.12)^{a}	18.13 (4.69)^{a}**
RK14	216.63 (43.27)^{a}	14.37 (4.60)^{a}**	219.19 (47.71)^{a}	15.18 (4.26)^{a}**
RK20	208.81 (41.00)^{a}	13.85 (4.42)^{a}**	207.17 (36.82)^{a}	14.35 (4.13)^{a}**
S	15.07 (2.09)^{b}	-----	14.43 (2.57)^{b}	-----

** significant difference with 1 at $p<0.05$
ns: non-significant difference at $p<0.05$
Similar letters in each column denote non-significant difference at $p<0.05$

3.3. Cross and multiple resistance

No statistical differences were observed between the GR50 and RFs of the two experiments in all cross and multiple resistance assays conducted. For clodinafop propargyl and sethoxydim, the RF of all resistant biotypes was significantly different from the value 1 in both experiments, whereas the RFs estimated for pinoxaden and mesosulfuron-methyl + iodosulfuron-methyl were statistically similar to 1. Also, there were no differences among the GR50s of the resistant biotypes in clodinafop propargyl and sethoxydim treatments, whereas the susceptible biotype had a statistically lower GR50 value compared with the resistant biotypes. In contrast, GR50s of the susceptible and resistant biotypes were statistically similar (Table 6). All haloxyfop-R-methyl -resistant biotypes had high resistance factors and maintained their dry weight at rates far more than the recommended field rate as a result of exposure to clodinafop-propargyl.

However, these resistant biotypes were partially resistant to sethoxydim and were unable to withstand pinoxaden and mesosulfuron-methyl + iodosulfuron-methyl herbicides and perished along with the susceptible biotype (Table 6).

In a study on *A. sterilis* subsp. *ludoviciana*-resistant biotypes from Fars province of Iran, resistance factors of the biotypes to clodinafop propargyl ranged from 1.76 to >47.04 (Sasanfar et al., 2017). The resistance factor of *A. fatua* biotypes collected from Mexico to pinoxaden was 3.58 (Torres-García et al., 2018).

Resistant biotypes had similar patterns regarding herbicide resistance. Although they showed high resistance to haloxyfop-R-methyl and clodinafop propargyl, exposure to sethoxydim almost halved the resistance factor among the biotypes. Thus, the biotypes had moderate resistance to sethoxydim herbicide. Furthermore, pinoxaden successfully suppressed growth of these biotypes, demonstrating their susceptibility to PPZ family herbicides. Since no resistance was observed to mesosulfuron-methyl + iodosulfuron-methyl among the biotypes as well, multiple resistance may not be attributed to the biotypes (Table 7).

For years, farmers of the region used the ACCase inhibiting herbicides as the sole option to control grass weeds in wheat fields (Gherekhloo et al., 2016). However, due to consecutive application of herbicides such as diclofop methyl, fenoxaprop-P ethyl and clodinafop propargyl resistance to ACCase family has the most cases observed in Iran (Heap, 2020). Cultivation of canola in rotation with wheat allowed the usage of another herbicide from ACCase family entitled haloxyfop-R-methyl, which became the main herbicide applied in canola fields of the region to control grass weeds for years (Gherekhloo et al., 2106). Haloxyfop-R-methyl and the three herbicides noted above belong to APPs, so canola-wheat rotation failed to increase the diversity in herbicide mode of action. Hence, the selection pressure on *A. sterilis* subsp. *ludoviciana* eventually led to further evolution of resistance.

Wild oat (*Avena* spp.) biotypes gathered from Australia with cross resistance to sethoxydim, clethodim and pinoxaden showed GR_{50} values of 281-1012, 9-23 and 12-69 g a.i. ha^{-1} with resistance factors of 3-10.5, 2.6-6.6 and 3.5-20, respectively (Ahmad-Hamdani et al., 2012). *A. fatua* oat biotypes gathered in USA were resistant to various herbicides such as pinoxaden, tralkoxydimm, imazamethabenz and flucarbazone (Keith et al., 2015). Cross resistance of *A. sterilis* subsp. *ludoviciana* to clodinafop propargyl, fenoxaprop-P ethyl, pinoxaden and tralkoxdim as well as multiple resistance to mesosulfuron-methyl + iodosulfuron-methyl has been observed in Greece (Papapanagiotou et al., 2019).

Table 6. Parameter estimates of *A. sterilis* subsp. *ludoviciana* response cross and multiple resistance assay. Values in parentheses represent standard error.

Herbicide	Parameter / Biotype	First experiment		Second experiment	
		GR_{50} (g a.i ha^{-1})	RF	GR_{50} (g a.i ha^{-1})	RF
Clodinafop-propargyl (APP)	RK5	472.94 (85.79)[a]	22.57 (5.98)[a]**	466.93 (89.03)[a]	21.79 (6.20)[a]**
	RK8	503.30 (82.57)[a]	24.02 (5.66)[a]**	515.29 (81.43)[a]	24.04 (6.54)[a]**
	RK12	519.67 (70.03)[a]	24.80 (5.55)[a]**	539.36 (91.33)[a]	25.17 (6.65)[a]**
	RK14	422.72 (75.02)[a]	20.17 (5.36)[a]**	425.53 (70.09)[a]	19.86 (5.30)[a]**
	RK20	415.08 (81.10)[a]	19.81 (5.88)[a]**	424.18 (76.30)[a]	19.79 (5.47)[a]**
	S	20.94 (2.97)[b]	-----	21.42 (3.48)[b]	-----
Sethoxydim (CHD)	RK5	444.12 (47.98)[a]	6.39 (0.24)[a]**	439.23 (43.96)[a]	6.22 (0.24)[a]**
	RK8	460.34 (48.71)[a]	6.62 (0.86)[a]**	455.14 (50.12)[a]	6.45 (0.86)[a]**
	RK12	466.63 (49.91)[a]	6.71 (0.82)[a]**	464.93 (45.94)[a]	6.59 (0.82)[a]**
	RK14	430.74 (43.68)[a]	6.19 (0.73)[a]**	426.33 (42.55)[a]	6.04 (0.73)[a]**
	RK20	423.35 (39.28)[a]	6.09 (0.63)[a]**	423.31 (44.12)[a]	6.00 (0.63)[a]**
	S	69.49 (3.36)[b]	-----	70.53 (5.10)[b]	-----
Pinoxaden (PPZ)	RK5	16.58 (4.88)[a]	0.98 (0.29)[a;ns]	15.90 (4.38)[a]	0.99 (0.27)[a;ns]
	RK8	13.72 (3.49)[a]	0.81 (0.21)[a;ns]	14.11 (4.44)[a]	0.88 (0.26)[a;ns]
	RK12	17.12 (3.20)[a]	1.01 (0.19)[a;ns]	17.83 (4.27)[a]	1.11 (0.27)[a;ns]
	RK14	14.67 (3.38)[a]	0.86 (0.20)[a;ns]	14.98 (4.57)[a]	0.94 (0.29)[a;ns]
	RK20	16.15 (4.41)[a]	0.95 (0.26)[a;ns]	14.13 (5.68)[a]	0.88 (0.35)[a;ns]
	S	16.91 (1.88)[a]	-----	15.94 (1.05)[a]	-----
Mesosulfuron-methyl+ iodosulfuron-methyl (ALS)	RK5	6.14 (1.32)[a]	1.03 (0.23)[a;ns]	6.03 (0.69)[a]	0.97 (0.11)[a;ns]
	RK8	5.41 (0.84)[a]	0.91 (0.13)[a;ns]	5.46 (0.81)[a]	0.88 (0.13)[a;ns]
	RK12	5.35 (1.14)[a]	0.90 (0.17)[a;ns]	4.98 (0.70)[a]	0.80 (0.12)[a;ns]
	RK14	6.08 (1.05)[a]	1.02 (0.19)[a;ns]	5.61 (0.72)[a]	0.90 (0.12)[a;ns]
	RK20	6.24 (0.99)[a]	1.05 (0.17)[a;ns]	6.73 (1.23)[a]	1.08 (0.31)[a;ns]
	S	5.94 (0.33)[a]	-----	6.18 (0.25)[a]	-----

** significant difference with 1 at $p<0.05$

ns: non-significant difference at $p<0.05$

Similar letters in each column in each herbicide treatment denote non-significant difference at $p<0.05$

Table 7. Resistance pattern of *A. sterilis* subsp. *ludoviciana* to selected herbicides according to Beckie and Tardiff (2012). Classification was done based on the RFs presented in Tables 5 and 6.

Herbicide / Biotype	Haloxyfop-R-methyl (APP)	Clodinafop-propargyl (APP)	Sethoxydim (CHD)	Pinoxaden (PPZ)	Mesosulfuron-methyl+ iodosulfuron-methyl (ALS)
RK5	H	H	M	S	S
RK8	H	H	M	S	S
RK12	H	H	M	S	S
RK14	H	H	M	S	S
RK20	H	H	M	S	S
S	S	S	S	S	S

S: non-resistant (RF<2); M: moderate resistance (RF=6-10); H: high resistance (RF>10)

3.4. Herbicide metabolism assay

The results showed that the effect of repetition was not significant and thus, the results of two experiment repetitions were similar. Also, only susceptible biotype was significantly affected by herbicide, and no other significant effects were observed (Table 8). Coleoptile length (percentage of control) of susceptible biotype plummeted significantly as a result of herbicide application compared to distilled water. Hence, herbicide metabolism has no role in evolution of resistance in the studied biotypes. Metabolism-related herbicide resistance may confer resistance or cross-resistance to current or new herbicides, so it is far more

difficult to control. To avoid this type of resistance, herbicides must be applied with care and at full rates (Yu and Powles, 2014).

Indirect study of herbicide metabolism using P450 inhibitors has been performed by researchers. Investigation on *Beckmannia syzigachne* (Steud.) Fernald biotypes resistant to ACCase and ALS inhibitors collected from China demonstrated that PBO had synergic effect on fenoxaprop-P-ethyl, confirming the involvement of metabolic resistance (Li et al., 2017). On the contrary, PBO and ABT had no significant effect on ACCase- resistant *Phalaris minor* Retz. biotypes (Gherekhloo et al., 2011).

Table 8. Analysis of variance for herbicide metabolism assay

SOV	df	Mean Squares					
		S	RK5	RK8	RK12	RK14	RK20
Repetition	1	8.02 ns	2.77 ns	11.11 ns	4.69 ns	14.69 ns	4.00 ns
Error I	4	25.94 ns	26.94 ns	10.22 ns	26.61 ns	9.19 ns	18.38 ns
Herbicide	1	73712.25 **	2.77 ns	11.11 ns	8.02 ns	20.25 ns	18.77 ns
Inhibitor	2	14.19 ns	8.77 ns	69.33 ns	3.86 ns	32.19 ns	22.86 ns
Herbicide×Inhibitor	2	9.08 ns	19.44 ns	3.11 ns	32.52 ns	9.25 ns	0.19 ns
Herbicide× Repetition	1	0.02 ns	25.00 ns	11.11 ns	20.25 ns	0.25 ns	9.01 ns
Repetition× Inhibitor	2	2.19 ns	24.11 ns	11.11 ns	4.69 ns	7.19 ns	5.58 ns
Repetition× Inhibitor× Herbicide	2	0.52 ns	6.33 ns	11.11 ns	20.25 ns	3.58 ns	12.58 ns
Error II	20	9.24	15.61	24.82	27.34	26.72	16.75
Total	35	----------					
CV (%)	---	6.07	4.20	5.31	5.56	5.49	4.37

ns and **: non significant and significant at p<0.01, respectively.

3.5. Molecular study

Allele specific PCR result for 5 mutation points of 1781, 1999, 2027, 2041 and 2078 demonstrated that in all resistant biotypes the fragment associated with Ile-2041-Asn mutation was amplified, suggesting the presence of Ile-2041-Asn allele. Since no other bands were observed at other studied points, Ile-2041-Asn is the only known resistance conferring mutation in the studied biotypes. Sequencing results served as a further confirmation that only Ile-2041-Asn mutation confers resistance in the biotypes (Fig. 3). Liu et al. (2007) used allele specific-PCR in a study on several ACCase-resistant *A. sterilis* subsp. *ludoviciana* biotypes, and detected various mutations on ACCase encoding gene. Allele specific-PCR was utilized to determine the mutation point on ACCase encoding gene endowing clodinafop propargyl resistance to little seed canary grass in India, which turned out to be Trp-2027- Cys and Ile-2041- Asn (Raghav et al., 2016). According to a study using allele specific-PCR method on *A. sterilis* subsp. *ludoviciana* populations gathered from the northern grain-growing region of Australia, Ile-2041-Asn mutation endowed resistance to fenoxaprop-P ethyl in one of the populations, whereas another population with the same mutation was resistant to both fenoxaprop-P ethyl and sethoxydim. In contrast to the results of the present study, this mutation did not endow haloxyfop resistance in *A. sterilis* subsp. *ludoviciana* (Liu et al., 2007). It must be

noted that the following research is a part of an extensive project which tends to monitor and trace resistant biotypes of major weed species in the whole Golestan province which will require processing of an enormous number of samples. Hence, a quick molecular-based test will be helpful to evaluate the resistance mechanism of the putative biotypes. Allele specific PCR method is cheaper than CAPS/dCAPS, does not need high quality DNA and has a relatively high accuracy. (Kadaru et al., 2008).

Fig. 3. Sequencing results for *A. sterilis* subsp. *ludoviciana* biotypes along with *A. myosuroides*. 2041 point is highlighted in red.

Wheat-canola rotation is very common in the region, and many farmers have adopted this cultivation pattern due to presence of numerous oilseed extraction companies as well as its nutritional value. *Avena sterilis* subsp. *ludoviciana* is currently the most troublesome weed infesting the region with devastatingly yield-reducing effect on both wheat and canola and farmers intend to suppress the growth of this obnoxious weed using agrochemical products.

Graminicides which are applicable in canola fields are more diverse than those of the wheat fields. Furthermore, they are safely applicable in canola which is dicot, so the farmers tend to apply these herbicides at higher rates which will lead to higher selective pressure imposed by herbicide on the weed. Hence, the evolution of resistance will be more rapid in canola fields compared to wheat.

According to the present study, *A. sterilis* subsp. *ludoviciana* pinoxaden was the only herbicide which successfully controlled *A. sterilis* subsp. *ludoviciana*. However, pinoxaden negatively affect canola due to phytotoxicity and its application may lead to losses in crop yield. Although susceptibility of *A. sterilis* subsp. *ludoviciana* to mesosulfuron-methyl + iodosulfuron-methyl indicates the feasibility of using ALS herbicides in wheat fields, their consecutive application will eventually lead to multiple-resistance, further complicating the problem. Moreover, ALS herbicides persist in soils for a relatively long time and affect the growth of the subsequent crop in the rotation (Papapanagiotou et al., 2019). Herbicides possessing

different modes of action to which this weed has not yet developed resistance may be used to wipe out both susceptible and resistant biotypes may be wiped out using an herbicide with a different mode of action, but it will serve as a new selective pressure eventually. The continuity of the selective pressure will gradually alter the relative frequency of resistant alleles in the population (Gherekhloo et al., 2012).

Changing the crop rotation pattern and increasing the diversity of herbicide mode of action would help to delay the further evolution of resistance in *A. sterilis* subsp. *ludoviciana*. Thus, further reliance on non-chemical and integrated weed management methods and debilitating the weed in competition with the crop may be a thoughtful option. Introduction of a row crop such as faba bean in crop rotation may decrease the frequency of resistant *A. sterilis* subsp. *ludoviciana* plants due to tilling. In the fields which are under canola-wheat rotation, it is also possible to use pinoxaden in wheat. In some parts of the region, preliminary studies have shown that the application of trifluralin in canola fields has successfully controlled ACCase-resistant *A. sterilis* subsp. *ludoviciana*. Also, cycloxydim and clethodim herbicides (which are currently not available in the region) can be tested in canola fields for controlling *A. sterilis* subsp. *ludoviciana*.

Conclusion

According to the results, *A. sterilis* subsp. *ludoviciana* biotypes were resistant to haloxyfop-R-methyl, clodinafop propargyl and sethoxydim. Currently, the frequency of this resistance in the studied region is low. However, it may become a serious challenge for canola growers of Golestan Province, Iran if not controlled properly. The chemical options for management of this weed in the region are currently limited. Thus, implementation of integrated weed management such as introduction of row crops such as faba bean in crop rotation and increasing the diversity of herbicide mode of action by cultivation of crops such as sugarbeet may prove helpful. In the fields which are under canola-wheat rotation, it is also possible to use pinoxaden in wheat. Also, trifluralin, cycloxydim and clethodim herbicides may be tested on *A. sterilis* subsp. *ludoviciana*.

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Highlights:

- **Resistance to haloxyfop-R-methyl was detected within the biotypes**
- **Biotypes were cross-resistant to clodinafop-propargyl and sethoxydim herbicides**
- **Ile-2041-Asn mutation is responsible for occurrence of resistance in the biotypes**
- **No metabolic resistance was observed in the biotypes**
- **Biotypes were susceptible to pinoxaden and mesosulfuron methyl+ iodosulfuron-methyl**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: