



## Article

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## FIRST REPORT OF MULTIPLE RESISTANCE IN *Galium aparine* TO ALS-INHIBITING AND AUXIN ANALOG HERBICIDES IN KERMANSHAH, IRAN

*Primeiro Relatório de Múltipla Resistência em Galium aparine a Herbicidas Inibidores da ALS e Análogos à Auxina em Kermanshah, Irã*

**ABSTRACT** - Catchweed Bedstraw (*Galium aparine*) is a problematic weed, which has become increasingly difficult to control with herbicides in Iran. The aim of this study was to screen selected putative-resistant populations of *G. aparine* for resistance to auxinic herbicides 2,4-D+MCPA and ALS-inhibiting herbicides sulfosulfuron, tribenuron-methyl, mesosulfuron-methyl + iodosulfuron-methyl-sodium. Populations of *G. aparine* were collected from different wheat fields in the west of Kermanshah, where herbicide-use pattern is typical for Iran. Herbicide resistance to premixed herbicide 2,4-D+MCPA was confirmed in several populations. More populations of *G. aparine* showed cross-resistance to ALS-inhibiting herbicides examined in this research. Some populations were found to have developed multiple resistant to both auxinic and ALS herbicides. Generally, the level of resistance to ALS-inhibitor herbicides was higher than that of auxin analog herbicides.

**Keywords:** 2,4-D+MCPA, iodosulfuron-methyl, mesosulfuron-methyl + iodosulfuron-methyl-sodium, sulfosulfuron, tribenuron-methyl.

**RESUMO** - *Galium aparine* é uma espécie de planta daninha problemática, que se tornou cada vez mais difícil de controlar com herbicidas no Irã. O objetivo deste estudo foi selecionar populações resistentes putativas de *G. aparine* quanto à resistência a herbicidas auxínicos 2,4-D + MCPA e herbicidas inibidores da ALS sulfosulfuron, tribenuron-methyl, mesosulfuron-methyl + iodosulfuron-methyl-sodium. Populações de *G. aparine* foram coletadas de diferentes campos de trigo no oeste de Kermanshah, onde o padrão de uso de herbicidas é típico para o Irã. Resistência herbicida ao herbicida pré-misturado 2,4-D + MCPA foi confirmada em várias populações. Mais populações de *G. aparine* mostraram resistência cruzada aos herbicidas inibidores da ALS examinados nesta pesquisa. Descobriu-se que algumas populações desenvolveram múltiplas resistências aos herbicidas auxínicos e ALS. Em geral, o nível de resistência aos herbicidas inibidores da ALS foi maior do que o dos herbicidas análogos à auxina.

**Palavras-chave:** 2,4-D+MCPA, iodosulfuron-methyl, mesosulfuron-methyl + iodosulfuron-methyl-sodium, sulfosulfuron, tribenuron-methyl.

## INTRODUCTION

*Galium aparine* L. from Rubiaceae family, is believed to have originated in Eurasia (Malik and Born, 1988). This weed species is found in temperate zones

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and tropics across the globe (Defelice, 2002). *Galium aparine* is widely distributed throughout the farmlands of Iran (MirKamali, 2007). Various characteristics, such as rapid seedling development, early flower initiation after a short period of vegetative growth, being semi-prostrate or climbing-ascending which result in adhering to or lying on adjacent crops make this weed a strong competitor (Wright and Wilson, 1987; Mennan and Zandstra, 2005). In addition, foliage and fruits of *G. aparine* is sticky which aids in its dispersal in short and long distances (Holm et al., 1977). These characteristics have made *G. aparine* a common and troublesome weed species in different cropping systems, particularly in winter wheat (Rassam et al., 2011).

*Galium aparine* could reduce the crop yield in winter wheat by competing for nutrients, light and water up to 20% on the average. Furthermore, results of a survey demonstrated that 10% of harvested grain from Iranian wheat fields are contaminated with seeds of *G. aparine* (MirKamali, 2007). This contamination occurs because weight and size of seeds of *G. aparine* is similar to that of wheat.

Like developed countries, using herbicides is the common and predominant weed control method across Iranian wheat fields (Deihimfard et al., 2007). Herbicides such as 2,4-D, MCPA, sulfosulfuron, tribenuron-methyl, mesosulfuron-methyl + iodosulfuron-methyl-sodium have been widely used to selectively control broadleaved weeds including *G. aparine* in wheat fields (Zand et al., 2007, 2010). However, herbicide failure has become the main constrain to the continued chemical control of *G. aparine* in wheat farmlands (Heap, 2018).

This study was conducted as many farmers and agricultural consultants have reported that control of *G. aparine* has become difficult with commonly applied herbicides in wheat fields of Kermanshah province, Iran. Therefore, the objective of this study was to determine if different populations of *G. aparine* are resistant across a range of most commonly used herbicides in wheat fields in the region.

## MATERIALS AND METHODS

**Seed collection:** Seeds of *G. aparine* were collected from 10 winter wheat fields located in Gorsefid [(34°12'N 45°50'E) (GR01, GR02, GR03)], Markazi [(34°08'N 45°54'E) (MA02, MA03, MA04)], Chele [(34°04'N 46°03'E) (CH01, CH02, CH03)], and Govaver [(34°01'N 46°19'E) (GO01)] districts from Gilane-Gharb city, Kermanshah province, Iran during June to July of 2016. The climatic conditions in selected districts was different representing a wide range of growing habitats in the western parts of Iran. All seed samples were from wheat fields where farmers reported a loss of control with commonly applied herbicides in the region (Table 1). Each population received at least three applications of selective herbicides during last five years. At each field, seeds were collected at maturity from 50 plants and pooled to make seed sample. Seed of a susceptible population (SP) was also collected from a wheat field (34°18'N 45°54'E), where there had been no known herbicide application.

**Preparing plants and treatment application:** Seeds of each population of *G. aparine* were stored under room temperature for two weeks until the start of experiments. For Herbicide resistance screening experiment, seedlings of *G. aparine* for applying herbicides were prepared by planting 150 seeds into 60 cm diam plastic containers containing a loamy soil comprised of 30% sand,

*Table 1* - Herbicides and rates applied for resistance screening of *Galium aparine* populations collected from Gilane-Gharb, Kermanshah, Iran

Chemical class	Mode of action	Active ingredient (s)	Trade name	Field dose (g a.i. ha <sup>-1</sup> )
Sulfonylureas	Inhibitors of acetolactate synthase	Sulfosulfuron	Apyros	20
Sulfonylureas	Inhibitors of acetolactate synthase	Tribenuron-methyl	Granstar	22
Sulfonylureas	Inhibitors of acetolactate synthase	Mesosulfuron-methyl + iodosulfuron-methyl-sodium + mefenpyr	Atlantis	18
Phenoxy-carboxylic acids (Phenoxy)	Synthetic auxins-disruptors of plant cell growth	2,4-d+MCPA	U46- D	1360

40% silt, and 30% clay with 0.50% total organic matter and a pH of 6.9. In all dose-response experiments, *G. aparine* seedlings were established by sowing 15 seeds into 25 cm diam plastic pots filled with the above-mentioned soil mixture. Seedlings were thinned to 3 per pot after emergence. *Galium aparine* seedlings were watered, fertilized, and maintained outdoors during February to June (coinciding with wheat growing season). Herbicide treatments were applied to plants at the 2 to 4 leaf growth stage using an experimental sprayer equipped with flat-fan nozzle delivering 180 L ha<sup>-1</sup>

**Herbicide resistance screening:** Each population of *G. aparine* was screened for resistance to the field rate of all four herbicides (Table 1) in comparison to a known susceptible population (SP). All of these herbicides are used routinely to control *G. aparine* and other broadleaved weeds of winter wheat fields in Iran and the region. Responses to herbicide treatment are expressed as percent survival, calculated as the proportion of the untreated control for each treatment replicate. Plant survival was assessed 28 days after treatment (28 DAT). If the growing point of treated was not affected by herbicide, then they were considered as alive. The criteria for determining the survival of plants treated with 2,4-D+MCPA, which is a premixed of auxin analog herbicides, was their capability to reach the reproduction stage. In addition, populations were nominated as resistant if e" 20% of the plants survive the field rate of a herbicide (Walsh et al., 2004).

**Dose-response experiments:** Results of herbicide resistance screening experiment showed that MA02 population has the highest level of resistance to tested herbicides in this study. Therefore, detailed dose-response experiments were conducted on this population as well as susceptible population (SP) using all tested herbicides for screening test (Table 1). Herbicides were applied as described above at 0, 1.0, 2.0, 4.0, 8.0, 16, 32, 64X rate, where X is the recommended dose of herbicide (Table 1). The shoot fresh weight was recorded 28 DAT and was expressed as a proportion of the untreated control for each population of *G. aparine*.

**Statistical analysis:** The design of each experiment was a randomized complete block with three replications, and each experiment was repeated once. Data for percentage of shoot fresh weight reduction were pooled over runs of the experiment because lack of run by treatment interaction. Data for herbicide resistance screening as well as shoot fresh weight reduction were subjected to ANOVA using SAS software (v. 9.1, SAS Institute, Cary, NC) and Fisher's Protected LSD test (P≤0.05) was used for means separation. To describe dose response of *G. aparine* populations, a nonlinear curve model (sigmoidal logistic, three parameters) using SigmaPlot software was fitted to data (Chandi et al., 2011):

$$Y = \frac{a}{1 + \exp\left(-\frac{x - x_0}{b}\right)}$$

where, Y is shoot fresh weight reduction (percentage of untreated control), a = the upper limit of Y; x is the herbicide dose, X<sub>0</sub>: GR<sub>50</sub> is the herbicide doses required to inhibit growth by 50% and b describes the slope of the curve in GR<sub>50</sub>.

## RESULTS AND DISCUSSION

**Resistance profile:** The herbicide-susceptible *G. aparine* population, SP, was controlled sufficiently with the recommended field doses of each of the herbicide commonly applied in wheat fields (Table 2). However, *G. aparine* populations exhibited survival to a range of herbicides applied at the field rate (Table 2). A high proportion (up to 100%) of populations collected from different districts survived auxin analog herbicides (2,4-d+MCPA), and ALS-inhibiting herbicide (sulfosulfuron, tribenuron-methyl, mesosulfuron-methyl+iodosulfuron-methyl). The MA02 population exhibited highest resistance level to 2,4-d+MCPA (60% survival), sulfosulfuron (85% survival), tribenuron-methyl (100% survival), and mesosulfuron-methyl+iodosulfuron-methyl (85% survival). In opposition, these values for susceptible population (SP) were 0, 7, and 10% survival, respectively. Hence, MA02 as well as some other collected populations exhibit multiple resistance across herbicides from two different modes of action and chemistries (sulfonylureas, auxin analog herbicides). To further explore the resistance status of the population showing the highest level of resistance to all tested herbicides in this study (MA02), detailed dose-response studies were conducted with this population.

**Table 2** - Response of susceptible and putative-resistant populations of *Galium aparine* to a range of herbicides of wheat fields applied at the field rate. Values are percent survival $\pm$ standard error

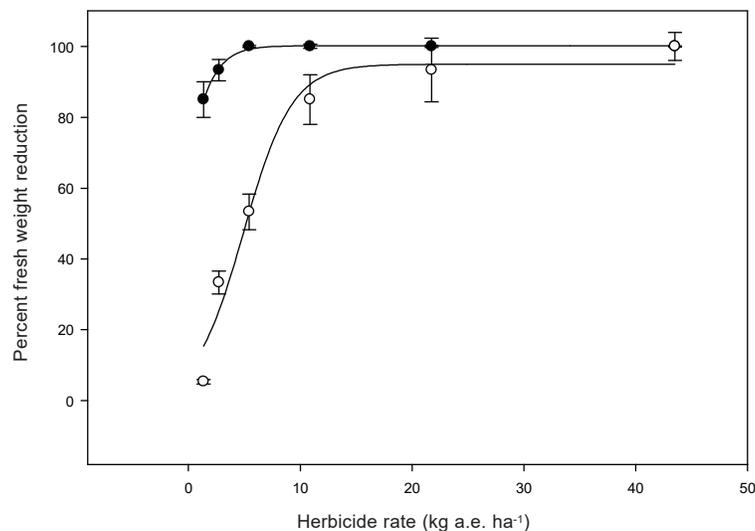
Herbicide	Collected population*										
	SP	GR01	GR02	GR03	MA02	MA03	MA04	CH01	CH02	CH03	GO01
	(%)										
2,4-d+MCPA	0	38 $\pm$ 5	38 $\pm$ 7	40 $\pm$ 9	60 $\pm$ 10.5	40 $\pm$ 3.7	52 $\pm$ 8	38 $\pm$ 9	32 $\pm$ 4.3	30 $\pm$ 2	28 $\pm$ 3.9
Sulfosulfuron	7 $\pm$ 2.3	56 $\pm$ 10	10 $\pm$ 3.8	60 $\pm$ 5.9	85 $\pm$ 6	62 $\pm$ 6.9	37 $\pm$ 3	10 $\pm$ 1.7	73 $\pm$ 7	50 $\pm$ 6	17 $\pm$ 4
Tribenuron-methyl	10 $\pm$ 3	63 $\pm$ 6.6	68 $\pm$ 11	87 $\pm$ 10	100 $\pm$ 3	77 $\pm$ 4.5	88 $\pm$ 8	87 $\pm$ 7	83 $\pm$ 6.7	67 $\pm$ 7.9	83 $\pm$ 10
Mesosulfuron-methyl + iodosulfuron-methyl	6 $\pm$ 2	62 $\pm$ 8.7	27 $\pm$ 7	75 $\pm$ 6	85 $\pm$ 9	30 $\pm$ 4	57 $\pm$ 8	60 $\pm$ 7.6	37 $\pm$ 5	60 $\pm$ 8	18 $\pm$ 3

\* SP, susceptible population; GR, Gorsefid; MA, Markazi; CH, Chele; GO, Govaver.

**Resistance to the auxin analog herbicides:** The GR<sub>50</sub> values for premixed auxin herbicides of 2,4-D+MCPA as well as all three ALS inhibitor herbicides in this study could not be calculated for the susceptible population as shoot fresh weight reduction was greater than 50% with the lowest tested rates.

**2,4-d+MCPA:** From the regression curve of shoot fresh weight reduction, it is obvious that MA02 population is highly resistant to 2,4-D+MCPA (Figure 1). Using 2,4-D+MCPA at 1360 g a.e. ha<sup>-1</sup>, the field application rate, reduced fresh weight (Table 3) which is approximately 4-fold of the recommended dose of the herbicide.

Applying 2,4-D+MCPA at 43.52 kg a.e. ha<sup>-1</sup>, 32 times the suggested field use rate, provided 100% shoot fresh weight reduction of *G. aparine*. In opposition, the susceptible population was controlled by 85% at the commercial rate (Figure 1). Heap and Morrison (1992) showed that resistant biotypes of wild mustard are 10- and 18-fold resistant to MCPA and 2,4-D, respectively, when compared to sensitive biotypes.



**Figure 1** - Shoot fresh weight reduction of susceptible population (●) resistant MA02 population (○) of *Galium aparine* in response to 2,4-D+MCPA.

Generally, target-site-based resistance (TSR) and nontarget-site-based resistance (NTSR) are two primary mechanisms of herbicide resistance in weeds (Yuan et al., 2007; Kreiner et al., 2017). Unlike ACCase- and ALS-inhibitors, auxinic herbicides ones, have low to no known natural variation for TSR alleles, which has resulted in a limited number of weeds with TSR. NTSR is now considered the only type of resistance identified to herbicide group O, namely Synthetic Auxins (2,4-D and MCPA) (Mithila et al., 2011; Délye et al., 2013). Due to involvement of multi-gene in mechanisms of, it is a greater danger to agriculture when compared with TSR. In addition, multi-herbicides resistance is frequently developed in NTSR (Ghanizadeh and Harrington, 2017).

**Table 3** - Parameter estimates and associated model statistics for the log-logistic dose response curves for shoot fresh weight reduction of *Galium aparine* in response to different herbicides

Model parameters				Herbicide
GR <sub>50</sub> ± SE (g a.i. ha <sup>-1</sup> )	R <sup>2</sup>	B	A	
4919 ± 705	0.98	2.169	94.98	2,4-D+MCPA
80.48 ± 15.19	0.97	78.89	90.80	Sulfosulfuron
130.30 ± 17.93	0.97	93.31	91.06	Tribenuron-methyl
82.0 ± 17.93	0.97	54.08	70.92	Mesosulfuron-methyl + iodosulfuron-methyl

From 1879 onwards, 2,4-D and MCPA (particularly 2,4-D) have been used extensively across Iran for control of broadleaved weeds in cereals (Deihimfard et al., 2007). In addition, *G. aparine* could be found in many Iranian cropping systems (Bischof, 1978). However, this is the first report of *G. aparine* exhibiting resistance to 2,4-D and MCPA. Furthermore, in some regions of Iran (e.g. the fields where the tested populations in this study were collected) have been treated twice. This duplication in herbicide usage occurs as maize is cultivated after harvesting of wheat and this herbicide mixture is applied in both of them. Hence, these populations have received extensive and long term applications of these herbicides. Despite this, the proportion of resistant individuals within the populations showing resistance to 2,4-D+MCPA remains relatively low. In addition, the reported cases of failure of this in controlling weeds is rare. Therefore, the rate of evolution of resistance to 2,4-D +MCPA within collected populations of *G. aparine* appears to be slow. These results revealed that prolonged use of auxinic herbicides has resulted in the development of resistance in *G. aparine*. However, resistance to this class of herbicides is relatively low, as it has been reported in a few number of weed species (Heap, 2018).

Broadleaved weeds, which are sensitive to auxinic herbicides generally have rare alleles imparting resistance, and potential for fitness penalties because of mutations conferring resistance. In addition, mode of action of auxinic herbicides is very complicated (Mithila et al., 2011; Busi and Powles, 2017). These reasons may explain the relatively low incidence of resistance to auxin analog herbicides (Pettersson et al., 2009). There are currently 34 weed species, which have evolved resistance to synthetic auxins globally. *G. aparine* have been reported to be resistant to this herbicide mode of action in China as well (Heap, 2018)

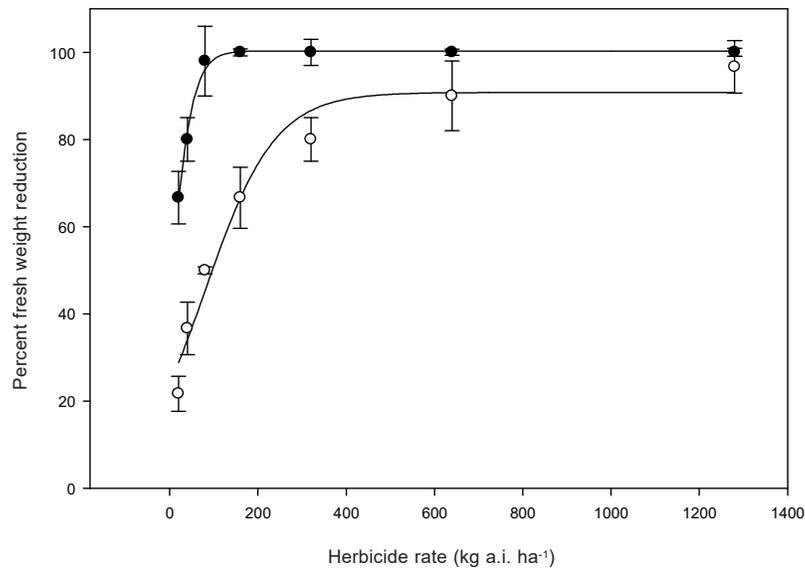
**Resistance to the ALS-inhibiting herbicide: Sulfosulfuron.** Dose-response study revealed that the MA02 population *G. aparine* was highly resistant sulfosulfuron while the susceptible population was controlled sufficiently with recommended dose (Figure 2). The treated population had an GR<sub>50</sub> of 80.48 g a.i. ha<sup>-1</sup> indicating a 4-fold increase over the recommended dose of herbicide (Table 3).

Applying sulfosulfuron at 20 g a.i. ha<sup>-1</sup>, the recommended rate, reduced shoot fresh weight of *G. aparine* by approximately 22% (Figure 2). The dose required for 97% shoot fresh weight reduction of MA02 by sulfosulfuron was 1280 g a.i. ha<sup>-1</sup> and complete reduction was not achieved even at highest herbicide doses (Figure 2).

**Tribenuron-methyl:** Tribenuron-methyl applied at rate of 22 g a.i. ha<sup>-1</sup>, the suggested field rate, failed to provide more than 17 and 76% control of resistant MA02 and susceptible population of *G. aparine*, respectively (Figure 3). As herbicide application rate increased, the level of *G. aparine* control also increased, although no treatment provided complete control (Figure 3).

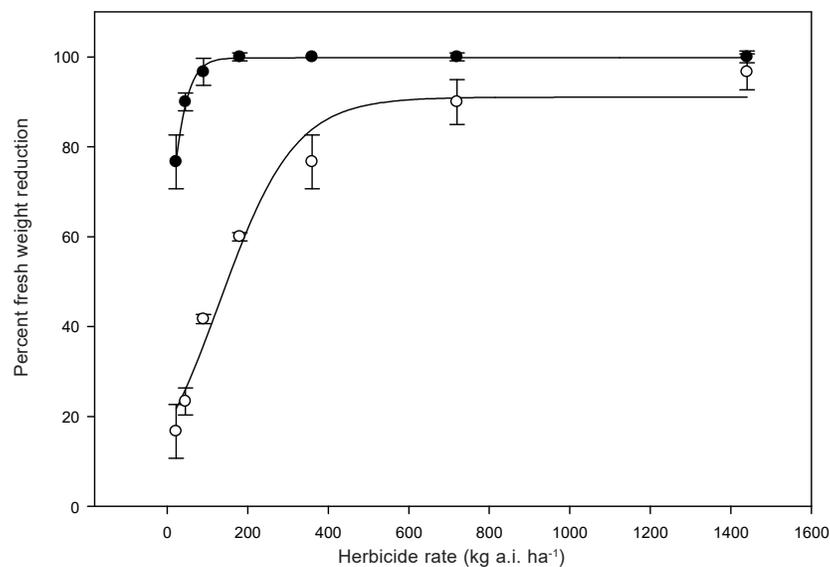
The herbicide required to reduce shoot fresh weight of *G. aparine* by 50% (GR<sub>50</sub>) was 130 g a.i. ha<sup>-1</sup> which is 5.9 times more than the recommended dose of tribenuron-methyl (Table 3). Tribenuron-methyl at 1440 g a.i. ha<sup>-1</sup> provided no more than 96% control of *G. aparine* (Figure 3).

**Mesosulfuron-methyl+iodosulfuron-methyl:** There was very little control of MA02 population of *G. aparine* with mesosulfuron-methyl + iodosulfuron-methyl, even at 36 g a.i. ha<sup>-1</sup>, or eight times the field rate (Figure 4). However, the fresh weight reduction of susceptible population was 70% by applying field rate of herbicide. The GR<sub>50</sub> value was 82 g a.i. ha<sup>-1</sup> which is nearly 4-fold the recommended dose of mesosulfuron-methyl + iodosulfuron-methyl (Table 3). The highest dose, 652 g a.i. ha<sup>-1</sup>, was not sufficient to cause more than 80% reduction in fresh foliage weight of *G. aparine* (Figure 4).



Error bars represent standard error of the mean.

**Figure 2** - Shoot fresh weight reduction of susceptible population (●) resistant MA02 population (○) of *Galium aparine* in response to sulfosulfuron.

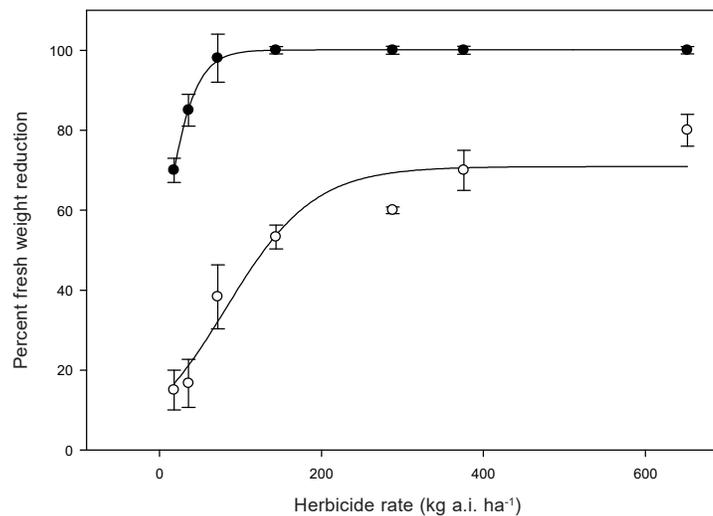


Error bars represent standard error of the mean.

**Figure 3** - Shoot fresh weight reduction of susceptible population (●) resistant MA02 population (○) of *Galium aparine* in response to tribenuron-methyl.

As it is clear from the results of the trials conducted in this study, there was cross-resistance in *G. aparine* to all three herbicides from sulfonylurea chemical family. Resistance to ALS-inhibitor occurred very fast. For the first time, prickly lettuce (*Lactuca serriola*) evolved resistance to chlorsulfuron following only five years of herbicide application (Tranel and Wright, 2002). Resistance to ALS herbicides represents the largest group of resistant weeds, which might be partially due to the presence of resistance alleles in many weedy species and the molecular structure of the target enzyme (Nakka et al., 2017).

Research has shown that *G. aparine* is now resistant to ALS inhibitors, including chlorsulfuron, iodosulfuron-methyl-sodium, mesosulfuron-methyl, thifensulfuron-methyl, triasulfuron, and tribenuron-methyl in Turkey and tribenuron-methyl in China (Heap, 2018).



Error bars represent standard error of the mean.

**Figure 4** - Shoot fresh weight reduction of susceptible population (●) resistant MA02 population (○) of *Galium aparine* in response to mesosulfuron-methyl + iodosulfuron-methyl.

Currently, wild mustard (*Sinapis arvensis*) and turnipweed (*Rapistrum rugosum*) are two broadleaved weed species infesting wheat and barley fields, which have evolved resistance to ALS-inhibitor herbicides in Iran (Gherekhloo et al., 2016). However, this is the first report of resistance of *G. aparine*, another important broadleaf weed of small grain cereals, to ALS inhibitors in Iran.

Resistance to both 2,4-D+MCPA and ALS inhibitors in *G. aparine* has created a serious threat to wheat production in Iran. Similarly, it is confirmed that wild radish (*Raphanus raphanistrum*) has evolved multiple-resistance to herbicides from auxin analog and acetolactate synthase-inhibiting herbicides in Australia (Walsh et al., 2004; Owen et al., 2015). Nearly all herbicides currently available for *G. aparine* control in wheat and barley fields in Iran are from either synthetic auxins or ALS-inhibitors.

As mentioned earlier, NTSR is the common type of resistance to Auxinic herbicides such as 2,4-D and MCPA (Mithila et al., 2011). Because NTSR can confer cross-resistance to herbicides that differ in mode of action, it probably promotes the evolution of NTSR to other common herbicides in the region (Ghanizadeh and Harrington, 2017). Thus, NTSR mechanisms selected by a herbicide can confer cross-resistance to herbicides with other modes of action, even those not yet applied in the field.

Hence, chemical rotations and/or tank mixing of different would not be useful in preventing the development of resistance herbicides with different modes of action. as a consequence, the only way to management of herbicide resistance under these circumstances would be adoption of non-chemical weed control methods.

It is well demonstrated that TSR is the predominant type of resistance to ALS-inhibitor herbicides in weeds (Deng et al., 2017). In essence, the development of target-site resistance depends on repeated usage of a single herbicide (or group of related herbicides). Therefore, the principal management strategy to reduce the risk of herbicide resistance development in weed targeted would be rotating or mixing herbicides with different modes of action. Furthermore, farmers need herbicides with new modes of action to control *G. aparine* and probably other broadleaved weeds present in wheat, particularly for short-term economic returns in intensively weed- infested fields. However, for long-term control of weeds adoption of non-chemical control measurements such as crop rotation, tillage and finally integrating all management options would be beneficial (Beckie, 2006).

In conclusion, the results of this study suggest that *G. aparine* populations have evolved multiple resistance to frequently used herbicides in wheat and barley fields in western Iran. Several populations exhibited resistance across different herbicides from ALS-inhibitor and auxin

analog. We have clearly established that at the population level there is multiple resistance across many herbicides of at least two modes of action. Future research will determine the mechanisms responsible for resistance to these herbicides. This first report of multiple-herbicide resistance *G. aparine* is very important in Iran as this weed infesting major small grain-production regions of Iran. The crop rotation and herbicide treatment of the fields were seed sample collected is a representative of whole of Iran. Hence, development of herbicide-resistance *G. aparine* probably will be repeated across Iran as well as other countries with identical situations.

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