EVALUATION OF RESISTANCE TO ACETOLACTATE SYNTHASE INHIBITING HERBICIDE IN WILD MUSTARD (Sinapis arvensis L.)

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ABSTRACT

To evaluate the mechanism and levels of herbicide resistance in Sinapis arvensis, a series of experiments were performed. Sequencing of ALS gene in S. arvensis sub-populations was carried out and ALS-based resistance (substitution of Asp by Glu) was detected at position 376 in this weed species. Further, it was reported that combined application of herbicide mesosulfuron + iodosulfuron can cause up to 50% reduction in dry weight (GR₅₀) of heterozygous resistant (FHR₃), homozygous resistant (FHR₂), and homozgyous susceptible (MHS₁) sub-populations and it was reported 2409, 603 and 289 g ha⁻¹, respectively. Further, resistance indices (RI) were reported 8.3 and 2 for FHR₃ and FHR₂ sub-populations, respectively. Based on LD₃₀, RI values were 8.5 and 4.5 for FHR₃ and FHR₂, respectively. Growth reduction occurred in the resistant homozygous when compared with the heterozygous sub-population in the presence of mesosulfuron + iodosulfuron.

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1 Introduction

From the ancestral time, farmers and researchers have made various efforts to control weeds. From last few decades use of herbicides increased drastically, however, despite the higher application of herbicides, sometime very limited success has been achieved in weed control (Mcgillion & Storrie, 2006; Gherekhloo et al., 2016). Further, continuous excess use of herbicide caused herbicide resistance in the crop weeds. According to Heap (2016) till today, total 471 weed species have developed resistance to herbicides. Globally, now in these days, among the herbicides which are currently used in agro-ecosystems, acetolactate synthase (ALS) inhibitor herbicides have been most widely used by farmers and researchers (Mallory–Smith et al., 1990).

ALS enzyme has catalytic effect on the branched chain amino acids, such as valine, leucine and isoleucine (Xu et al., 2010; Cui et al., 2011). ALS enzyme is the target-site of different chemical families such as sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinylthiobenzoates and sulfonylaminocarbonyl-triazolinones (Cruz-Hipolito et al., 2013). In case of target site based herbicide resistance, herbicide cannot bind to the enzyme catalytic site (Pang et al., 2002; Duran-Prado et al., 2004; Duggleby et al., 2008; Powles & Yu, 2010; Jian et al., 2011). Common amino acid substitutions in the ALS gene that confirmed target site resistance and provide protection against herbicide damage are Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654 (Yu & Powles, 2014; Cross et al., 2015). Consequently, substitution of certain amino acids within the herbicide binding area can cause resistance to some herbicides, but not all of them (Yu et al., 2012).

Assessment of herbicide resistance are often carried out using based on the comparison of growth and survival of the plants suspected to be resistant or susceptible to different range of herbicide doses. Moreover, recently, uses of molecular methods also facilitate the detection of herbicide resistance mechanism (Kaundun & Windass, 2006; Delye et al., 2013). Herbicide resistance mechanisms divided in two groups of target-site and non-target-site resistance. The vast majority of the resistance cases to ALS inhibitors reported in the literature are target-site resistance (Tranel et al., 2011; Wiersma, 2012). Identifying target-site resistance mechanism is vital for understanding, diagnosing and managing herbicide resistance.

So, molecular approaches include sequencing (Corbett & Tardif, 2006), enable deciphering the genetic basis of resistant (Duran-Prado et al., 2004; Duggleby et al., 2008; Breccia et al., 2013; Ochogavia et al., 2014; Tranel et al., 2014). Therefore, in this study, it was investigated that whether target-site mutation confers resistance to ALS inhibitor herbicide with two active ingredient (mesosulfuron + iodosulfuron) in wild mustard (S. arvensis) or not, and, if so, the resistance levels for each homozygous/heterozygous mutation were also determined.

2 Materials and Methods

2.1 Plant materials

Two resistant and one susceptible sub-population of S. arvensis were used in this experiment. Seeds of mesosulfuron + iodosulfuron resistant S. arvensis plants collected from different wheat fields in Firoozabad, Fars Province, Iran. The seeds were separately planted in pots already filled with peat. At the 3–4 leaf stage, seedlings from FHR1, FHR2 and MHS1 sub-populations were treated with mesosulfuron-methyl (10 g/L) + iodosulfuron-methyl-sodium (2 g/L) at commercial rates of 0, 187, 375, 1500 and 3000 mg ha\(^{-1}\) (Atlantis, 40 OD, Bayer, Crop Science, Germany), utilizing a moving-boom with 8004 Tee-Jet nozzles sprayer delivering herbicide at a rate of 400-L water ha\(^{-1}\) at a pressure of 200 kPa. The pots were kept outside in growing season. Survival rate and dry weight were assessed 4 weeks after treatment.

2.2 ALS gene sequencing

ALS sequencing was obtained for each sub-population of FHR1, FHR2, and MHS1. Total genomic DNA was extracted from each plant of sub-populations by utilizing genomic DNA isolation kit (Denazist, Mashhad, Iran). Fragments of the ALS gene that included the regions of domains A, B, C, D and E were amplified. Changes in these domains can cause target-site resistance to ALS inhibitor herbicides. ALS primers were designated from S. arvensis (Accession numbers: FJ861277.1 and FJ655877.1). The specific primers used were, SAR1F-5'-CTA TGT CCT ACG TTA TGA GCC-3' and SAR1R-5'-TCG AGC TTT CCC GTG ACA CG-3'. SAR2F-5'-GTA ACG ACG AGT TGT CTC TGC-3' and SAR2R-5'-TCC AAC AGG TAT GTA CCT GG-3'. These primers were used to detect any amino acid changes in the ALS gene and related to ALS resistance to S. arvensis. Sequencing of the purified genomic DNA was performed in the Medical University, Shiraz, Iran.

2.3 Statistical analysis

All data presented here are mean values of four replicates. For herbicide dose-response assay, the dose required to kill 50% of the population (LD\(_{50}\)) or cause 50% dry weight reduction (GR\(_{50}\)) was calculated by non-linear regression using a three-parameter log-logistic model which fitted better than the other models (Xu et al., 2010; Cui et al., 2011).

\[
y = a/[1 + (x/x_0)^b]\]

Where, \(a\) is the upper limit, \(b\) is the slope of the curve, \(x\) is a constant and \(x_0\) represent to herbicide dose that reduces survival and dry matter by 50 percent. Resistance index (RI) of S. arvensis to mesosulfuron + iodosulfuron was computed as \(\text{IR}_{50}\) (FHR\(_2\) or FHR\(_1\)/IR\(_{50}\) (MHS)). \(\text{LD}_{50}\) and \(\text{GR}_{50}\) indices were analyzed based on sigmaplot 12.0.
Table 1 The resistance level of different *S. arvensis* sub-populations from Iran, to Mesosulfuron + iodosulfuron by dose-response experiment.

| Sub-populations | LD$_{50}$ (g ha$^{-1}$) | a    | b    | $R^2$ | RI |
|-----------------|--------------------------|------|------|-------|----|
| FHR$_3$         | 3160                     | 98.83| 5.42 | 0.99  | 8.5|
| FHR$_2$         | 1664                     | 96.69| 5.59 | 0.99  | 4.5|
| MHS$_1$         | 371                      | 98.02| 5.17 | 0.99  | -  |

| Sub-populations | GR$_{50}$ (g ha$^{-1}$) | a    | b    | $R^2$ | RI |
|-----------------|--------------------------|------|------|-------|----|
| FHR$_3$         | 2409                     | 100.21| 3.53 | 0.99  | 8.3|
| FHR$_2$         | 603                      | 103.23| 2.25 | 0.99  | 2  |
| MHS$_1$         | 289                      | 99.36 | 3.18 | 0.99  | -  |

LD$_{50}$ and GR$_{50}$ effective dose of Mesosulfuron + iodosulfuron causing 50% reduction in survival and dry weight respectively; RI, ratio of LD$_{50}$ or GR$_{50}$ values relative to the susceptible sub-population. RR is homozygous Glu-376 resistant sub-population, RS is heterozygous (Asp/Glu-376) resistant sub-population and SS is homozygous (Asp-376) susceptible sub-population.

3 Results and Discussion

3.1 Plant dose-response assays

The plant dose-response assay study revealed the GR$_{50}$ values for FHR$_3$, FHR$_2$ and MHS$_1$ sub-populations and it was reported 2409, 603 and 289 g ha$^{-1}$ for mesosulfuron + iodosulfuron application respectively (Table 1). Further, RI values were reported 8.3 and 2 for FHR$_3$ and FHR$_2$ sub-populations respectively (Table 1 and Figure 1). Based on GR$_{50}$ and RI values, it can be concluded that FHR$_3$ and FHR$_2$ sub-populations have low to moderate resistant against mesosulfuron + iodosulfuron, respectively (Figure 3). Moreover, no MHS$_1$ sub-population growth at the recommended dose of mesosulfuron + iodosulfuron was identified (Figure 1).

![Figure 1](image-url) Effect of Mesosulfuron + iodosulfuron on dry weight of the homozygous resistant (Glu-376) FHR$_3$ (dot line), heterozygous resistant (Asp/Glu-376) FHR$_3$ (solid line), and homozygous (Asp-376) susceptible MHS$_1$ (dash line) *S. arvensis* sub-populations from Froozabad, Iran. Error bars represent standard error of three to four replicates.

The similar trend was reported for the LD$_{50}$ values and it was reported 3160, 1664 and 371 g ha$^{-1}$ for the FHR$_3$, FHR$_2$ and MHS$_1$ sub-populations respectively on the application of mesosulfuron + iodosulfuron (Table 1 and Figure 2). RI values were 8.5 and 4.5 for FHR$_3$ and FHR$_2$, respectively. Growth of the FHR$_2$ sub-population was reduced (Figure 1) and resulting in 1.3 fold higher LD$_{50}$ than the GR$_{50}$ values (Figure 1, Figure 2 and Table 1).

Moreover, based on LD$_{50}$ homozygous *Raphanus raphanistrum* for Asp-376-Glu were highly resistant to sulfonylurea herbicide chlorsulfuron (Duhoux et al., 2015). In addition, some studies emphasis that the homozygous Asp-376-Glu resistant plants treated with ALS herbicides are weaker than 122-Tyr, 197-Ser and 574-Leu in different resistant weeds (Warwick et al., 2005; Whaley et al., 2007; Ashigh et al., 2009; Duhoux et al., 2015).
Table 2 ALS sequence data. Substitution at position 376 resulted in amino acid changes in resistant *S. arvensis* sub-populations; and all other substitutions are neutral.

| Nucleotide Polymorphisms | Amino acid | Amino acid no. | codon | population |
|--------------------------|------------|----------------|-------|------------|
|                          | Ser | Thr | Leu | Gly | Asp | Pro | Thr | Pro | Val | Gly | Leu | Glu |
| MHS1                     | TCT | ACT | CTG | GGA | GAT | CCT | ACA | CCT | GTT | GGA | TTG | GAG |
| FHR2                     | C   | G   | T   | C   | A   | C   | C   | T   | A   | G   | G   |
| FHR3                     | C   | G   | C   | T   | C   | C   | A   | C   | T   | G   | G   |
| Arabidopsis              | T   | T   | C   | A   | T   | A   | C   | T   | A   | G   | G   |

Amino acid positions are indicated by ALS gene from Arabidopsis thaliana (sathasivan et al. 1990). Nucleotide base are indicated by A=adenine, C=cytosine, G=guanine, T=thymine. Domain C: amino acids 115 to 133; Domain A: amino acids 191 to 203; Domain D: amino acids 205 to 210; Domain B: amino acids 573 to 576; Domain E: amino acids 651 to 655.

Figure 2 Effect of Mesosulfuron + iodosulfuron on survival of the homozygous resistant (Glu-376) FHR2 (dot line), heterozygous resistant (Asp/Glu-376) FHR3 (solid line), and homozygous (Asp-376) susceptible MHS1 (dash line) *S. arvensis* sub-populations from Froozabad, Iran. Error bars represent standard error of three to four replicates.
Figure 3 ALS genes sequencing results showing (a) the GAC codon for Asp-376 in homozygous susceptible (MHS₁) sub-population, (b) the GAC/GAA codons for Asp/Glu-376 in heterozygous resistant (FHR₁) sub-population and (c) the GAA codon for Glu-376 in homozygous resistant (FHR₂) sub-population.

Unfortunately, the mechanism of this pathway in ALS homozygous Glu-376 is not clear (Duhoux et al., 2015). Therefore, as stated above, homo/heterozygous Asp-376-Glu mutation in two resistant sub-populations (FHR₁ and FHR₂) represents a complex situation for resistance to mesosulfuron + iodosulfuron or even other sulfonylurea herbicides.

3.2 ALS gene sequencing

Sequences 1601 and 1572 bp obtained from ALS gene sequencing were compared with the sequences in the genbank using Blast software (Blast http://www.ncbi.org). Fragments sequenced have the highest percentage of similarity (99%) with ALS genes, and this is the reason for the accurate sequencing of S. arvensis (Table 2). Finally, specific primer pairs of SAR1F/SAR1R, and SAR2F/SAR2R were isolated. After the blast of resistant and susceptible genes of S. arvensis,
point mutation at site 376 resulted in the substitution of aspartate by glutamate (Table 2).

Sequences isolated from ALS gene in different sub-populations of *S. arvensis* and Arabidopsis are shown in Table 2. All sequences were based on the Arabidopsis sequence (Table 2). At sites 175, 178 181, 212 and 248, nucleotide changes occurred at susceptible (MHS) and homozygous resistant (FHR) sub-populations of *S. arvensis*, but did not change the amino acid (synonymous substitution). Consequently, they cannot be a factor of herbicide resistance. In another experiment, comparison of 18 resistant and susceptible populations of *S. arvensis* collected from Canada, demonstrated mutations unrelated to change of amino acid (Warwick et al., 2005).

Even though the RHR and FHR sub-populations have the same mutation and identical ALS sequences. Furthermore, these changes were observed only at the beginning of the ALS gene. When the PCR fragments between R and S samples were compared, 2 homozygous susceptible (Figure 3a), 5 heterozygous resistant (Figure 3b), and 5 homozygous resistant individuals were revealed (Figure 3c). The results obtained demonstrate that ALS-based mutation (Asp-376-Glu) confers resistance in *S. arvensis* sub-populations (FHR and RHR) and are in line with the results reported in the literature (Whaley et al., 2007; Zhang et al., 2011; Duhoux et al., 2015). Evolved ALS target-site resistance is most common in six positions (Ala-122, Pro-197, Ala-205, Asp-376, Tsp-574 and Ser-653), although other target positions have been reported in rare cases (McCourt et al., 2006).

**Conclusion**

Tribenuron-methyl resistance in *S. arvensis* sub-populations of Iran wheat fields can be attributed to ALS target-site (Asp-376-Glu) mutation resulting in homo/heterozygous sub-populations. Nevertheless, in the presence of herbicide, unknown factors other than target-site resistance mechanism can mediate the response of homozygous resistant sub-population (FHR) to decrease growth rate. This is a problematic issue that has the potential to cause shift in our understanding and management of weed resistance. Finally, with regards to the point of evolutionary-based of herbicide resistance, we have to consider both molecular and biological aspects.

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**Conflict of interest**

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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