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Characterization of triazine resistant biotypes of junglerice [Echinochloa colona (L.) Link.] found in Iran

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Abstract

Echinochloa colona (L.) Link. (junglerice) is one of the most troublesome weed species in Iranian sugarcane fields. Monoculture with repeated use of herbicides with the same mode of actions in sugarcane fields enhanced the rate of herbicide resistance. Six suspected resistant junglerice biotypes (R1, R2, R3, R4, R5 and R6) were obtained from sugarcane fields in Khuzestan province, Iran. Dose-response and gene sequencing studies were conducted to elucidate the basis for ametryn and metribuzin resistance in these biotypes. The EC_{50} values of R1, R2, R3, R4, R5, R6 and S biotypes were 11.95, 13.11, 7.35, 2.54, 26.57, 2.46 and 0.034 and 11.61, 27.72, 27.13, 5.54, 66.65, 1.12 and 0.11 mg ai L^{-1} of ametryn and metribuzin in pregerminated seed assay, respectively. The ED_{50} values of R1, R2, R3, R4, R5, R6 and S biotypes were 6212, 3046, 4396, 1709.2, 9427, 491.58 and 106.53 and 1667.2, 1195, 830.18, 672.43, 4458.7, 140.55 and 54.68 g ai ha^{-1} of ametryn and metribuzin in the whole plant assay, respectively. Resistance factor (Rf_{50}) of the R-biotypes varied from 72.35 to 781.47 and 10.18 to 605.09 in seedling assays and from 4.62 to 88.64 and 2.57 to 81.54 in the whole plant assay of ametryn and metribuzin, respectively. DNA sequence analysis of the *psb*A gene, which is the target site of PS II inhibiting herbicides, revealed that two nucleotide changes (A to G) at position 232 and 286 conferred two amino acid substitutions from serine to glycine at residue 264 in the R-biotypes (except R4) and glycine to serine at residue 282 in the S- biotype.

Keywords: Gene sequencing, photosystem II, point mutation, *psb*A gene.
Abbreviations: Ametryn_N^2-ethyl-N^4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine; DNA_deoxyribonucleic acid; D1_a kind of protein; EC_{50} _effective concentration; ED_{50}_effective dose; Rf_{50}_resistance factor; Metribuzin_4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5 (4H)-one); *psb*A_a kind of gene.

Introduction

Since the first report in 1970 on triazine-resistant weed, common groundsel (*Senecio vulgaris* L.), herbicide resistance has been reported to most herbicide classes among 210 Weed species (Heap, 2013; Ryan, 1970). Herbicide resistance can be conferred by several mechanisms, including reduced target site sensitivity, target site amplification, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site (Devine and Shukla, 2000). Menendez et al. (2007) reported three mechanisms of resistance to photosystem II inhibiting herbicides. First, a lack of herbicide absorption and/or translocation may prevent the herbicide from reaching the target site at lethal dose. The second is enhanced herbicide metabolism due to an enzyme system. Third is the mutation in the target sites that results in a reduced affinity of the herbicide for the target. Until now, most researchers believed that the mechanism of herbicide resistance in plants is often an altered target site or due to the metabolism of the herbicide (Park and Mallory-Smith, 2005). Target site resistance usually occurs as a single nucleotide change in the gene encoding of the target protein. Therefore, it is easy to identify them and many examples of target site resistance to herbicides (Preston, 2009). The most common mechanism of resistance to triazine herbicides is a mutation in the *psb*A gene, which encodes the D1 protein, leading to an alteration at the site of the protein where triazine binding occurs (Foes, 1998; Gadamski et al., 2000; Park and Mallory-Smith, 2005; Tian and Darmency, 2006; Menendez et al., 2007; Perry et al., 2012). Ametryn and metribuzin, triazine herbicides, are registered in Iran for pre and post emergence control of broadleaf and some grass weeds in potato (*Solanum tuberosum* L.), soybean (*Glysine max* L.), sugarcane (*Saccharum officinarum* L.), tomato (*Lycopersicon esculentum* L.), alfalfa (*Medicago sativa* L.) and garden asparagus (*Asparagus officinalis* L.). In southwest Iran, Khuzestan province, sugarcane is one of the most commonly grown crops, with junglerice among the most common weeds. Triazine herbicides inhibit photosynthesis in susceptible plants by blocking photosystem II (PS II) transport reactions (Elefthrohorins et al., 2000; Tharayil-Santhakumar, 2003). These herbicides block the transfer of electrons from Q_A (the electron donor), to Q_B (the mobile electron carrier) (Devine and Shukla, 2000; Elefthrohorins et al., 2000; Tharayil-Santhakumar, 2003). They function biochemically by displacing to the plastoquinone (PQ)-binding site on the D1 protein in the PS II reaction center of the photosynthetic electron transport chain (Abbaspoor and Streibig, 2007). The D1 protein is a heterodimer thylakoid-membrane spanning protein and heart of the PSII reaction center, coded by the *psb*A gene (sundby et al., 1993). Binding

Table 1. Summary of EC_{50} values for the fresh weight in dose-response of resistant (R) and susceptible (S) biotypes of *Echinochloa colona* to ametryn and metribuzin in the pregerminated seed assay.

Herbicide	Biotype	Lower limit (SE)	Upper limit (SE)	Slope (SE)	EC_{50} (SE)	Lack of fit test (5%)	Rf_{50}
Ametryn	R1	0.025 (± 0.0023)	0.082 (± 0.002)	0.63 (± 0.08)	11.95 (± 2.35)	0.12 (NS)[a]	351.47
	R2	0.024 (± 0.0023)	0.059 (± 0.002)	1.11 (± 0.77)	13.11 (± 4.10)	0.08 (NS)	385.59
	R3	0.021 (± 0.005)	0.055 (± 0.004)	0.38 (± 0.12)	7.35 (± 6.28)	0.61 (NS)	216.18
	R4	0.014 (± 0.0013)	0.051 (± 0.002)	0.73 (± 0.11)	2.54 (± 0.57)	0.06 (NS)	74.71
	R5	0.023 (± 0.0023)	0.07 (± 0.0014)	0.85 (± 0.12)	26.57 (± 5.16)	0.69 (NS)	781.47
	R6	0.015 (± 0.0025)	0.053 (± 0.0021)	0.33 (± 0.05)	2.46 (± 1.19)	0.14 (NS)	72.35
	S	0.012 (± 0.0009)	0.062 (± 0.0012)	0.38 (± 0.03)	0.034 (±0.005)	0.20 (NS)	-[b]
Metribuzin	R1	-	0.078 (± 0.003)	0.20 (± 0.02)	11.61 (± 4.98)	0.07 (NS)	105.55
	R2	0.016 (± 0.005)	0.070 (± 0.002)	0.43 (±0.08)	27.72 (±10.42)	0.11 (NS)	252
	R3	-	0.054 (± 0.003)	0.22 (±0.03)	27.31 (±12.08)	0.14 (NS)	248.27
	R4	0.019 (± 0.002)	0.067 (± 0.002)	0.61 (± 0.10)	5.54 (± 1.15)	0.052 (NS)	50.36
	R5	-	0.065 (± 0.003)	0.19 (± 0.03)	66.56 (±31.97)	0.85 (NS)	605.09
	R6	0.016 (± 0.001)	0.053 (± 0.002)	0.43 (± 0.08)	1.12 (± 0.33)	0.16 (NS)	10.18
	S	0.012 (± 0.001)	0.042 (± 0.001)	0.39 (± 0.05)	0.11 (± 0.03)	0.29 (NS)	-

[a]NS: non-significant at the 5% level. [b]Parameter not estimated. Rf_{50}, EC_{50} of the resistant biotype / EC_{50} of the susceptible biotype.

the PS II herbicides to the D1 protein inhibit electron transport. Therefore, It cause two major consequences: (1) a shortage of reduced NADP, which is required for CO_2 fixation; and (2) the formation of free radicals (H_2O_2, OH^-, Chl^3, etc.) which cause photo oxidation of important molecules in the chloroplast, e. g., chlorophylls, unsaturated lipids, etc (Devine and Shukla, 2000). Eberlein et al. (1992) reported the first occurrence of metribuzin resistance for Powel amaranth (*Amaranthus powelli* S. Watson) in potato in Idaho. In Europe, for the first time, Elefthrohorins et al. (2000) reported metribuzin resistance in redroot pigweed (*Amaranthus retroflexus* L.) and common lambsquarters (*chenopodium album* L.) found in potato grown in the Nevrokopi, Greece. In Asia, Zand (2009) reported atrazine and metribuzin resistance for junglerice biotype found in sugarcane grown in Iran (Heap, 2013). However, the triazin resistance reported in this study is the first documented report from Asia. The appearance of junglerice populations with different degrees of resistance to other herbicides has been reported previously in Latin America (Fisher et al., 1993; Valverde, 2007), Arkansas (Hoagland et al., 2004) and Spain (Kim et al., 2000). Several detection methods of resistant weed species have been developed, including whole plant assays, petri dish assays, chlorophyll fluorescence, leaf disk flotation, pollen germination and molecular assays (Kim et al., 2000). Among these methods, petri dish and whole plant assays are rapider and cheaper than others for detecting herbicide resistance. The objectives of this research were to (1) investigate whether junglerice biotypes developed resistant biotypes to triazine herbicides (2) compare the response of suspected resistant biotypes with susceptible biotype to triazine (3) determine cross-resistance of the resistant biotypes to triazine herbicides (4) determine the mechanisms of resistance and (5) compare the biotype response to triazine herbicides in petri dish and green house.

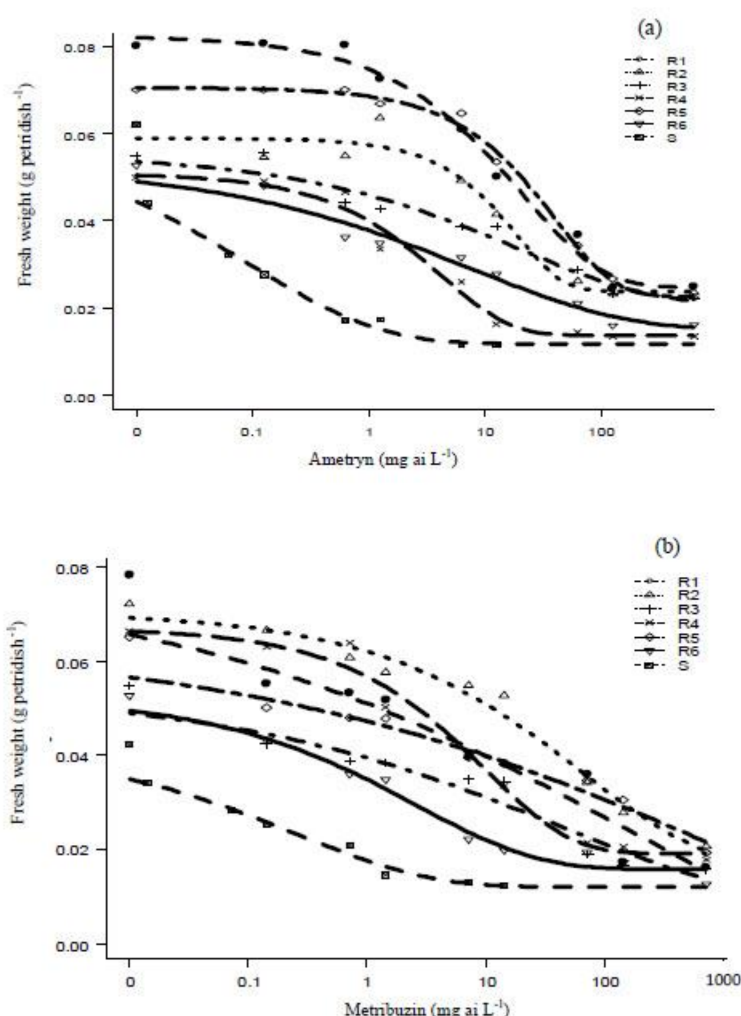

Fig 1. Dose-response for fresh weight of resistant (R) and susceptible (S) biotypes of *Echinochloa colona* to ametryn (a) and metribuzin (b) in the pregerminated seed assay.

Results and discussion

Pregerminated seed assay

Fourteenth days after ametryn and metribuzin treatments, a clear difference in symptoms was observed between the R- and S- biotypes. Ametryn and metribuzin affected the fresh weight of S- and R-biotypes of junglerice differentially. Fresh weight of the S-biotype was inhibited at 0.034 to 12.5

Table 2. Summary of ED_{50} values for the fresh weight in dose-response of resistant (R) and susceptible (S) biotypes of *Echinochloa colona* to ametryn and metribuzin in the whole plant assay.

Herbicide	Biotype	Lower limit (SE)	Upper limit (SE)	Slope (SE)	ED_{50} (SE)	Lack of fit test (5%)	Rf_{50}
Ametryn	R1	-[a]	1.47 (±0.05)	0.27 (± 0.03)	6212 (± 1671)	0.95 (NS)[b]	58.41
	R2	-	1.83 (± 0.05)	0.29 (± 0.03)	3046 (± 663.74)	0.054 (NS)	28.64
	R3	-	1.65 (± 0.05)	0.29 (± 0.04)	4396 (± 1183.8)	0.88 (NS)	41.34
	R4	-	2.11 (± 0.05)	0.37 (± 0.03)	1709.2 (± 306.5)	0.59 (NS)	16.07
	R5	-	1.48 (± 0.04)	0.27 (± 0.03)	9427 (± 2505)	0.89 (NS)	88.64
	R6	0.96 (± 0.15)	1.98 (± 0.07)	0.35 (± 0.09)	491.58 (± 398.6)	0.96 (NS)	4.62
	S	-	1.49 (± 0.04)	0.63 (± 0.06)	106.35 (± 14.42)	0.09 (NS)	-
Metribuzin	R1	-	1.39 (± 0.06)	0.37 (± 0.05)	1667.2 (± 455.9)	0.06 (NS)	30.5
	R2	-	1.76 (± 0.05)	0.34 (± 0.03)	1195 (± 262.43)	0.33 (NS)	21.85
	R3	0.49 (± 0.12)	1.63 (± 0.04)	0.37 (± 0.06)	830.18 (± 436.6)	0.73 (NS)	15.18
	R4	-	2.15 (± 0.06)	0.28 (± 0.02)	672.43 (± 155.5)	0.07 (NS)	12.3
	R5	-	2.08 (± 0.06)	0.29 (± 0.03)	4458.7 (± 1029)	0.08 (NS)	81.54
	R6	0.68 (± 0.03)	1.99 (± 0.04)	0.59 (± 0.07)	140.55 (± 25.55)	0.63 (NS)	2.57
	S	-	1.48 (± 0.04)	0.76 (± 0.07)	54.68 (± 5.92)	0.06 (NS)	-

[a]Parameter not estimated. [b]NS: non-significant at the 5% level. Rf_{50}, ED_{50} of the resistant biotype / ED_{50} of the susceptible biotype.

mg ai L^{-1} and 0.11 to 14.3 mg ai L^{-1} of ametryn and metribuzin, respectively. But those of the R-biotypes were only slightly decreased at the highest ametryn and metribuzin concentrations (Fig. 1). The biotype R5, with highest EC_{50}, was the most resistant to both ametryn and metribuzin, followed by R2, R1, R3, R4, and R6 of ametryn and R2, R3, R1, R4 and R6 of metribuzin. The EC_{50} values of R5 were 26.57 and 66.56 mg ai L^{-1} of ametryn and metribuzin, respectively, which is about 781.47 and 605.09 times higher than the EC_{50} of S-biotype of ametryn and metribuzin, respectively (Table 1). The responses of R-biotypes were different from those of ametryn and metribuzin. So, the EC_{50} values of R1, R2, R3, R4, R5, R6 and S biotypes were 11.95, 13.11, 7.35, 2.54, 26.57, 2.46 and 0.034 and 11.61, 27.72, 27.13, 5.54, 66.65, 1.12 and 0.11 mg ai L^{-1} of ametryn and metribuzin in pregerminated seed assay, respectively (Table 1). The Rf_{50} values of the R1, R2, R3, R4, R5, R6 and S biotypes were 351.47, 385.59, 216.18, 74.71, 781.47 and 72.35 of ametryn and 105.55, 252, 248.27, 50.36, 605.09 and 10.18 of metribuzin, respectively (Table 1). These results clearly demonstrated that ametryn and metribuzin R- and S-biotypes could be differentiated easily by the pregerminated seed assays. These findings are in agreement with the results of Kim et al. (2000) who found that discrimination between R- and S- junglerice biotypes was possible on the basis of GR_{50} values for fresh weight in pregerminated seed assay with fenoxaprop. Furthermore, the method could detect effectively cross resistance of junglerice to PS II-inhibiting herbicides. Totally, the R- biotypes were categorized into very high (> 100) (R1, R2, R3, R4) and high (10-100) (R5 and R6) resistance to ametryn and metribuzin according to Beckie and Tardif (2012).

Whole plant assay in pots

For both ametryn and metribuzin, differences in fresh weight between R- and S-biotypes were readily apparent (Fig. 2). The ED_{50} values of the R1, R2, R3, R4, R5 and R6 biotypes were 6212, 3046, 4396, 1709.2, 9427 and 491.58 g ai ha^{-1} compared with 106.35 g ai ha^{-1} of the S-biotype of ametryn. The Rf_{50} of the R1, R2, R3, R4, R5 and R6 biotypes were 58.41, 28.64, 41.34, 16.07, 88.64 and 4.62 of ametryn, respectively. The ED_{50} values of the R1, R2, R3, R4, R5 and R6 biotypes were 1667.2, 1195, 830.18, 672.43, 4458.7 and 140.55 g ai ha^{-1} compared with 54.68 g ai ha^{-1} of the S-biotype of metribuzin. The Rf_{50} values of the R1, R2, R3, R4,

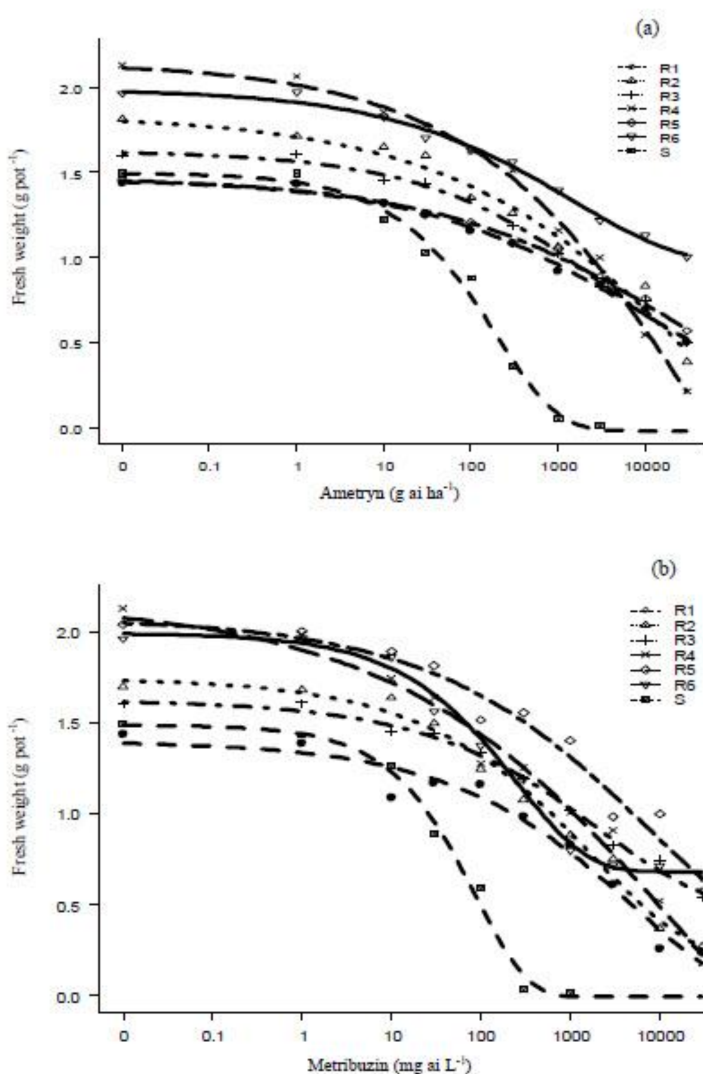

Fig 2. Dose-response for fresh weight of resistant (R) and susceptible (S) biotypes of *Echinochloa colona* to ametryn (a) and metribuzin (b) in the whole plant assay.

R5 and R6 were 30.5, 21.85, 15.18, 12.3, 81.54 and 2.57 of metribuzin, respectively. The biotype R5, with highest ED_{50}, was the most resistant to ametryn and metribizin, followed by R1, R3, R2, R4 and R6 of ametryn and R1, R2, R3, R4, and R6 of metribuzin (Table 2). The R- biotypes were categorized into high (10-100) (R1, R2, R3, R4 and R5) and Low (2-5) (R6) resistance to ametryn and metribuzin according to Beckie and Tardif (2012). The results showed that metribuzin was more potent than ametryn. These findings are in agreement with the results of Devine and shukla (2000), who stated that most s-triazine resistant biotypes show a high level of cross-resistance to other s-triazine herbicides and a lower level of resistance to as-triazinones (e. g., metribuzin). Also, Gadamski et al. (2000) reported resistant biotypes of Canadian horseweed (*Conyza canadensis* (L.) Cronquist) and barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.), both with the 264 serine to glycine mutation survived at least 30 times greater atrazine rate than susceptible biotypes. Moreover, our resistant biotypes showed cross-resistant to triazine and triazinone herbicides in this experiment. Similarly, Menendez et al. (2007) revealed that resistant biotypes of cheatgarss (*Bromus tectorum* L.) showed cross-resistance to other groups of triazine herbicides, the hierarchy of resistance level being methoxy-s-triazines ≥ chloro-s-triazines > methyl-thio-s-triazines > cis-triazines.

Gene sequencing

The DNA sequence analysis of the *pab*A gene, identified a 335-bp coding region, which encoded 187-297 amino acid residues in D1 protein. Thirty four single nucleotide differences were identified among the R- and S-biotypes thirty two of which produced no change in the encoded amino acid sequence. But, the nucleotide change (A to A/G) at position 232 and (A to G) at position 286 provided an amino acid substitution from serine to glycine in the R-and S-biotypes. It should be noted that these positions were corresponding to amino acid codon 264 and 282 in cheatgrass (Park and Mallory-Smith, 2005) and slim amaranth (*Amaranthus hybridus* L.) (Hirschberg and McIntosh, 1983). Also, we identified Ser at residue 282 in the S-biotype, whereas, amino acid at this residue was Gly in susceptible biotype of cheat grass and slim amaranth. It could be concluded that this mutation could have played a role in herbicide sensitivity in our susceptible biotype.

Totally, five of R-biotypes (including R1, R2, R3, R5 and R6) that were sequenced exhibited a Ser_{264} to Gly substitution in the *psb*A gene. One resistant biotype (R4) did not possess the *psb*A mutation at position 264. Herbicide resistance in this biotype can be conferred by several mechanisms other than mutation, including reduced target site sensitivity, target site amplification, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site. It should be noted that the R-biotypes (except R4) were heterozygous at this position. Therefore, they were categorized as heterozygous-resistant (R/S) and (S) biotypes.

Comparison between experimental methods

Junglerice resistance to triazine herbicides can be determined with the pregerminated seed, whole plant and molecular assays. Seed germination assay using aqueous emulsions of herbicides in petri dishes have been widely and successfully used for detecting resistance of wild oat (*Avena* spp.) to triallate (O’ Donovan et al., 1996), acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides (Murray et al., 1996; Rastgoo et al., 2010; Zand et al., 2010) and iodosulfuron+mezosulfuron (Aghajani et al., 2010), black grass (*Alopecurus myosuroides* Huds.) to aryloxyphenoxypropionic acid (Letouze and Gasques, 1998), junglerice to propanil and fenoxaprop (Kim et al., 2000), rye grass (*Lolium* spp.) to aryloxyphenoxypropionate (Letouze and Gasques, 1998; Tal et al., 2000) and littleseed canary grass (*Phalaris minor* Retz.) to aryloxyphenoxypropionate (Tal et al., 2000; Elahifard et al., 2008; Gherekhloo et al., 2008). These methods have advantages and disadvantages in comparison with each other. The pregerminated seed assay is much more rapid than the whole plant assay because it requires 4-6 weeks before concluding observations. The pregerminated seed assay allows identification of R-biotypes within 6-14 days depending on herbicide type. Second, in the whole plant assay, the discrimination between lowly and highly resistant biotypes requires spraying of a range of doses. With the pregerminated seed assay, however, only one dose of herbicide allows the distinction of resistant biotypes. Third, the pregerminated seed assay is a simple, rapid, low-cost compared to the whole plant assay which is space and time- consuming. Nevertheless, this method has a main limitation in that the petridishes containing seedlings incubated in incubators or germinators don’t have enough space. An important finding was that the resistance factor (Rf_{50}) values of biotypes in the pregerminted seed assay were higher than that in the whole plant assay. Whilst, a large number of researchers maintained that the Rf_{50} values of resistant biotypes were lower than the whole plant assay. As Tal et al. (2000) stated seedling reactions could be different from whole plant reaction in pot because of being different conditions in petri dish. The temprature in which the seed bioassay is conducted may be especially important because it can affect the activity of triazine herbicides (McMullan and Nalewaja, 1990). On the other hand, because of being effective on seedlings growth, PSII-inhibiting herbicides requiring photosynthesis initiates and seedlings grow sufficiently. Therefore, for detecting triazine resistance in pregerminated seed assay measuring fresh weight is better than coleoptiles or radical length. The whole plant assay is a common method and more accurate than pregerminated seed assay. Because non soil bioassay methods could not detect the probable resistance effects on herbicide action compared with the pot assay, performing the pot assay is necessary in order to validate the results of other bioassay methods. The molecular test is much more rapid than the pregerminated seed assay but it is more expensive than the pregerminated seed and whole plant assay. In addition, we couldn’t understand if R-plant phenotype’s resistance herbicide mechanism is the target site-based or not. Therefore, it could be suggested that it’s necessary that researcher carry out other methods to validate their findings. Herbicide resistance in weed biotypes may be conferred by several possible mechanisms such as modified target site, enhanced herbicide metabolism, absorption or translocation and membrane depolarization (Letouze and Gasquez, 1999). In a modified target site mechanism because of changed binding site, herbicide resistance to high doses can occur in these biotypes. In our research, the R-biotypes (R5, R1, R2, R3 and R4) showing a high level of resistance to the experimental herbicides may have modified target site. Consequently, the lowly R-biotypes (such as R6) might resist by mechanisms other than an insensitive triazine biotype, involving an enhanced capacity to detoxify these herbicides. But, it should be noted that the R-biotypes contain individuals that carry mutation. Overall, according to the test, these populations may be composed of seeds lowly, moderately, highly and

Table 3. Herbicides and rates used in dose-response studies with resistant (R) and susceptible (S) junglerice biotypes.

		Herbicide rates	
Herbicide	Assay	R	S
	Pregerminated seed	mg ai L^{-1}	
Ametryn		0, 0.125, 0.625, 1.25, 6.25, 12.5, 62.5, 125, 625	0, 0.0125, 0.0625, 0.125, 0.625, 1.25, 6.25, 12.5
Metribuzin		0, 0.143, 0.715, 1.43, 7.15, 14.3, 71.5, 143, 715	0, 0.0143, 0.0715, 0.143, 0.715, 1.43, 7.15, 14.3
	Whole plant	g ai ha^{-1}	
Ametryn		0, 1, 10, 30, 100, 300, 1000, 3000	0, 1, 10, 30, 100, 300, 1000, 3000, 10000, 30000
Metribuzin		0, 1, 10, 30, 100, 300, 1000	0, 1, 10, 30, 100, 300, 1000, 3000, 10000, 30000

very highly resistant to triazine herbicides that triggered different responses to the herbicide application. Finally, it could be concluded that the results of the pregerminated seed, the whole plant and molecular assay were in agreement with each other.

Materials and methods

Seed source

Suspected resitant and susceptible seeds of junglerice to ametryn and metribuzin were collected from sugarcane fields and adjacent areas of Karun Agro-Industry Inc., Shushtar, southwestern of Iran in 2010 - 2011 growing season. These biotypes were named according to their biotype status and abbreviated as follows: R1, R2, R3, R4, R5, R6 (suspected resistant biotypes) and S (susceptible biotype). All the collected seeds (R- and S-biotype) were stored at room temperature (25 ^{o}C).

Pre-germinated seed assay

The seeds pregerminated in petri dishes contained a single layer of Wathman No.1 filter paper in a seed germinator at 28± 2 C in a 16/8 hours (light/dark) photoperiod and 60% relative humidity for 72 h. Different ranges of herbicide rates were used for the R- and S- biotypes because of the difference in dose-response between biotypes (Table 3). These experiments were carried out by placing 5 pre-germinated seeds in a 7-cm-diam petri dish containing one piece of filter paper and 5 ml of distilled water or solutions having different concentrations of ametryn and metribuzin. The petri dishes were placed in the germinator. The fresh weight of seedlings was measured 14 days after treatment (DAT).

Whole plant assay

The seeds pregerminated in petri dishes at 28 ± 2 ^{0}C in 16/8 hours (light/dark) photoperiod for 72 h. The pregerminated seeds were sown in pots (with 10 cm diameter) containing loam: sand 2:1 mixture (v/v). Pots were transferred to a greenhouse and grown at 25 ^{0}C and 20 ^{0}C day and night temperature, respectively, with artificial light to provide a 16-h photoperiod. Pots were irrigated regularly to avoid any moisture stress. Ten days after planting (DAP), they were thinned to two seedlings per pot. Twenty days after weed emergence, seedlings of the pots were subjected to the post emergence herbicide application. Different ranges of herbicide rates were used for the resistant and susceptible biotypes because of the difference in dose-response among biotype (Table 3). Nonionic surfactant 0.25% (v/v) was

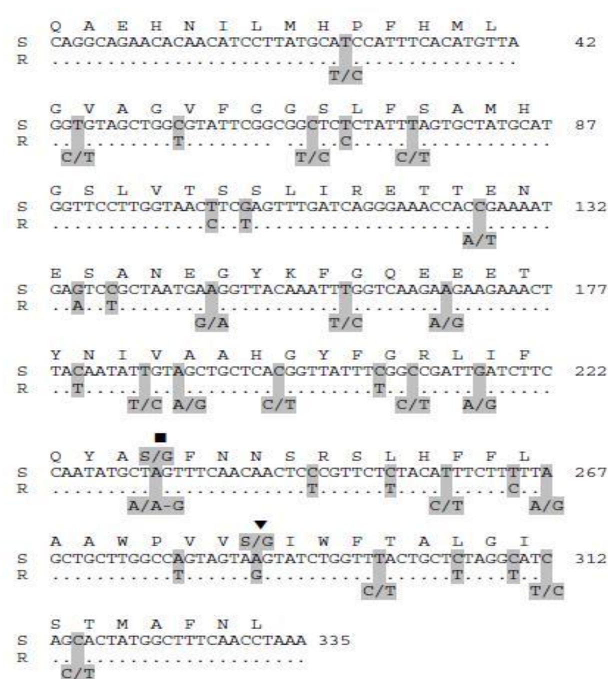

Fig 3. Comparison of nucleotide and deduced amino acid sequences coding for the *psb*A gene of the D1 protein from susceptible (S) and resistant (R) junglerice biotypes. Shaded nucleotides indicate 34 modifications in R-biotypes in comparison S-biotype that 32 of them are silent mutation. The nucleotide difference (A/ A-G) and (A/G) at position 232 and 286 are indicated by closed square (■) and closed triangle (▼), followed by the corresponding change in the amino acid serine to glycine observed among the S- and R-biotypes, respectively. GenBank accession number is JX885723 for the *psb*A gene in the susceptible biotype.

applied with the herbicide at the time of spraying. The sprayer was calibrated to deliver 220 L ha^{-1} at pressure of 2 atm. The aboveground biomass was harvested 28 days after treatment (DAT) and weighed.

Gene sequencing

DNA sequencing was used to recognize the molecular basis resistance. Leaf tissue (about 150 mg) from plant of each of the junglerice biotypes was used for DNA extractions following the methods described by Doyle and Doyle (1987). The region of the *psb*A gene in the template DNA was amplified using the polymerase chain reaction (PCR). Primers for the *psb*A were the same as those used by Foes et

al. (1998). Amplifications of *psb*A gene for each plant were carried out as separate reactions. Amplification reactions contained ~25 ng of total DNA, 0.4 µM of each of two primers, 1 unit of Taq polymerase (Cinnagen Co., Iran), $Mgcl_2$ 1 mM, dNTPs 0.2 mM, PCR buffer (10x) 2.5 µL, in a final volume of 25 µL. These reactions were subjected to a 4-min incubation at 94 ^{0}C; 35 cycles of 0.5-min at 94 ^{0}C, 0.5-min at 60 ^{0}C and 0.75-min at 72 ^{0}C; and 5-min incubation at 72 ^{0}C. The PCR products were fractioned through 1.2% agarose gel and SB (1x) buffer. Green viewer (Pars Toos Co., Iran) was used for dying gel. PCR products were sequenced by Bioneer Co. and analyzed by Sequencer software (BioEdit v7.0.5).

Statistical analysis

All experiments were conducted two times in a completely randomized design with three replications for the pregerminated seed assay and four replications for the whole plant assay. The data were analyzed using a nonlinear regression model and R software (drc add on packeges) (Knezevic et al., 2007), the four- parameter gompetrz (Eqn 1) was fitted to the data to describe the responses of the biotypes to herbicides:

$$Y = c + (d - c) \exp\{-\exp\{b(\log(x) - e)\}\} \quad (1)$$

Where e is EC_{50} or ED_{50}, the upper limit (d) is the response when dose is zero, and the lower limit (c) is the response when dose is maximum. The parameter b denotes the relative slope around e and x is the dose.
In cases where c=0, the three parameter model (Eqn 2), with the lower limit equaling zero, was refitted to the data (Knezevic et al., 2007):

$$Y = d \exp\{-\exp\{b(\log(x) - e)\}\} \quad (2)$$

The resistance factor (Rf_{50}), which is the ratio of the EC_{50} or ED_{50} of the resistant population to the EC_{50} or ED_{50} of the susceptible population was estimated as an index in order to compare the resistance levels of the tested populations.

Conclusion

The results obviously demonstrated that ametryn and metribuzin R- and S-biotypes could be discriminated by the pregerminated seed, whole plant and the molecular assays. The results confirmed that the mutation Ser_{264} confers a sufficient level of resistance to ametryn and metribuzin. Our hypothesis is that field evolved triazine-resistant junglerice populations would be comprised of individuals carrying a diverse range of resistance-conferring mutations and that individuals would be heterozygous or homozygous for one or any two different mutations. Thus, it can be said that resistance to triazine herbicides depends on the specific resistant allele(s), the homo/heterozygous status of individual plants for the specific resistant allele(s), and combinations of different resistant allele(s) plus herbicide doses. In the present study we only sequenced portions of the *psb*A gene. Therefore, it is possible that there are other *psb*A sequence polymorphisms between the resistant and sensitive biotypes that affect herbicide sensitivity. We suppose that the rate and pattern of herbicide use by Karun Agro-Industry Inc. authorities must have provided suitable selection pressure to select these mutants. It is likely that we would have found more *psb*A mutants if we had screened more biotypes. Therefore, monitoring is needed to avoid contamination of non-infested fields and also limiting movement of resistant seeds to minimize resistance risk in other fields. Managing herbicide-resistant weeds requires a good understanding of the mechanism of resistance involved in these populations of junglerice for various herbicides. The key components of resistance management are the use of mixtures and sequences of herbicides with different modes of action. Moreover negative cross-resistance can be used as a potent tool for controlling triazine-resistant weeds. We believe that more populations of junglerice must be studied to determine specific mutations. Further research is also required to determine the role of these mutations on plant responses to herbicide application.

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