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Regualr Article

Evaluation of Rigid Ryegrass (*Lolium rigidum*) accessions for clodinafop-propargyl Resistance using bioassay in Petri dish and pot

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Three rigid ryegrass accessions with possible resistance to clodinafop-propargyl, an acetyl-CoA carboxylase (ACCase) inhibitor (group 1) herbicide were identified in Fars province fields. Greenhouse studies and seed bioassay experiments were conducted to confirm clodinafop-propargyl resistance in *Lolium rigidum* L. In Petri dish seed bioassay test were determined the coleoptiles length of accessions (as % of untreated control), ID50 (dosage required to inhibit coleoptile length by 50%), the degree of resistance in S and R accessions. In greenhouse experiments to determine the degree of resistance, accessions were exposed to clodinafop-propargyl at 4-leaf stage. Four weeks after spraying was calculated the dry weight of accessions (%), plant survival of each accession (% of untreated control) and evaluation was performed according to EWRC. Then, the responses of accessions exposed to 0.25 to 32 times the recommended dosage of clodinafop-propargyl was measured and the degree of resistance in S and R accessions was identified. Results of bioassay and greenhouse studies revealed that in both experiments and for parameters measured FR1 accession showed more resistance than the other accessions, followed by FR7 and S accessions, respectively. So these two methods tested can be used to help assess the degree of resistance and reached the same result with two methods.

Keywords: acetyl-CoA carboxylase (ACCase), degree of resistance, recommended dosage.

Ryegrass (*Lolium rigidum* L.) is an annual problematic weed in wheat fields that can often a large expand in high density (Karimi, 2008). Use of herbicides is the most common means of weed control in wheat. Today, herbicides are one of the most essential inputs in modern farming systems and a considerable portion of the production is due to the use of herbicides (Lane Crooks et al, 2005).

Among the herbicides labeled for use in wheat, diclofop and clodinafop has for decades been the most effective in controlling grass in many wheat production areas. The use of herbicides in the Fars province has been reported more than 10 years (Deihimfard and Zand, 2004). Continual use of acetyl-CoA carboxylase ACCase; inhibiting herbicides, aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD), in wheat fields has resulted in the evolution of resistance in grass weed species (Beckie et al., 2000). Overall, resistance to herbicides has become as a one of the main research in recent years over the world (De'lye, 2005). Since their introduction in the late 1970s, 28 grasses weed biotypes including *Alopecurus myosuroides*، *Avena sp., Digitaria sp., Echinocloa sp., Hordeum sp., Phalaris sp.,* and *setaria sp.* (Zand and Baghestani, 2002).

Prior to 1998, there have not been any herbicide-resistant weeds in crop-growing regions of Iran (Zand et al., 2006).The first case of resistance to ACCase inhibiting herbicides was reported in wild oat (*Avena* spp.) in Khozestan Province, Iran , in 2005, 24, 12 and 10 years after the introduction of diclofop , phenoxaprop and clodinafop, respectively (Zand et al., 2006). According to literature by the end of 2009, 331 weed biotypes of 189 different species (113 Dicot and 76 monocot species) are resistant to the different herbicides (Heap, 2008).

Herbicide resistance in weeds occurs usually due to continuous use of the same herbicide or repeated application of herbicides with the same site of action (Powles and Shaner, 2001). ACCase inhibitors are selective herbicides that widely used early post-emergence in many crops to control perennial and annual grassy weeds in grass and broadleaf crops (Friesen et al., 2000). Continuous use of these herbicides has led to the emergence of resistant biotypes to herbicides (Heap and Knight, 1986). The first case of resistance to ACCase herbicides was reported in ryegrass in South Australia (Powles et al., 1997). Resistance to this herbicide group in ryegrass and wild oat threatens cereal production in countries such as Australia, Canada, Chile, France, Saudi Arabia, North Africa, Spain, England and the United States (Banakashani et al., 2006).

Repeated applications of herbicides has resulted in the evolution of resistance population particularly *Lolium rigidum* in Australia. Experiments have confirmed multiple resistance in *Lolium rigidum to* aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD), Carbamates, Sulfonylureas, Imidazolinones, chloro-acetamides, Dinitroanilines, which each of these groups of herbicides inhibit specific enzymes (Powles and Shaner, 2001). Detection of Glyphosate-resistance rigid ryegrass in California show that even after the development of herbicide-resistant crops, herbicides resistance management strategies are still important in the world (Simarmata and Donald, 2005).

Zand et al. (2008) evaluated the efficacy of ACCase-inhibiting herbicides labeled in Iran to control R and S rigid ryegrass biotypes. They found that clodinafop propargyl, cycloxydim, pinoxaden (at doses 450 and 600ml ha^{-1}), iodosulfuron+mesosulfuron (shovalia), iodosulfuron+mesosulfuron (Atlantis), and isoproturon+diflufenican in controlling S accessions and sethoxydim, iodosulfuron + mesosulfuron (atlantis), pinoxaden (at dose 600 ml ha^{-1}), isoproturon+diflufenican in controlling R accessions showed good performance. Also, diclopopmethyl, sethoxydim and chlosulfuron controlled S accessions partly.

Several reports exist on Resistance to ACCase inhibitor herbicides in *Lolium rigidum,* hence, use of alternative herbicides or other control strategies to avoid herbicide resistance development are undeniable. Otherwise, resistance can develop faster in this species, where the herbicide-resistant weed management strategies maybe more expensive. The objective of this research was to determine the rate and level of resistance in *Lolium rigidum* biotypes collected from Fars province to clodinafop propargyl herbicide.

Materials and methods

Plant material

Two suspected rigid ryegrass accessions collected from Fars province field were evaluated with a susceptible accession. Susceptible accession seeds collected from sites with no Clodinafop propargyl use history and suspicious accessions seeds to clodinafop-propargyl resistance were gathered from sites that were exposed to Clodinafop propargyl applications up to five year.

Greenhouse studies

Dose-response experiments.

Greenhouse experiments were conducted as a factorial in a completely randomized design with three ryegrass accessions. Ryegrass accessions seeds were planted 1.5 cm in 9 cm-diam plastic pots clay/sand/manure mixture in a 2:1:1 ratio (by vol.). The plants were grown in a greenhouse at 30/25 C day/night temperatures with a 14-h photoperiod. Clodinafop propargyl were applied to rigid ryegrass accessions at dosages proportional to 0, 0.25, 0.5,1, 2, 4, 8, 16, 32 times the recommended dosages on label. Ingredient active (ai) used for these dosages were 0, 16, 32, 64, 128, 256, 512, 1024 and 2048 gr ai ha $^{-1}$ (Zand et al., 2008). All rigid ryegrass plants were sprayed at two to three-leaf stages with moving-nozzle sprayer. Plants were evaluated for visual injury at two times on a scale of 0 to 100%, with 0 equal to no visible injury and 100 equal to dead plants. The remaining plant placed on pots were counted 4 weeks DAT Plant survival (%) was evaluated 30 DAT compared with control. Then plant were harvested and aboveground biomass were weighed after being oven-dried at 75 C for 48 and wet and dry weight of each accession determined compared with control.

Petri dish bioassay study

In order to access a quick way to evaluate the resistance of ryegrass accessions resistant to clodinafop-propargyl, a Petri dish bioassay was carried out in two steps including determination of discriminating dose for susceptible biotype and dose-response experiment for determination the resistance degree of suspected and susceptible biotypes.

Determination of discriminating dose for susceptible biotype:

In most studies, discriminating dose is determined in term of 50 inhibition of trait studied. According to Beckie et al. (2000) a discriminating dose is the minimum herbicide dose that makes the maximum vertical difference between dose–response curves of R and S biotypes and that causes at least 80% control of the S biotypes. Seeds of susceptible accession were germinated under required conditions. Soon after radicle emergence, germinated seeds of S accession were transformed to 9-cm-diam plastic Petri dishes containing two layers of filter paper. Ten seeds of S biotype were placed on each Petri dish with three replications for each treatment. Clodinafop-propargyl treatments were 0, 1, 10, 100 and 1000 ppm. Herbicide solutions were prepared based on dosage. Eight milliliters of clodinafop-propargyl solution was added in each petri dish and eight milliliters of distilled water was placed in control. Then petri dishes incubated in germination chambers at 25/15 C (day/night) for 7 d (the first five days in absolute dark and two days remaining in absolute light). Length of the primary shoot was measured after 7 days and was calculated as percentage of control. After clarification the herbicide dosage that cause 50% inhibition of shoot growth in susceptible accession, this dosage was applied on all ryegrass accessions in separate study.

Determination of resistance rate

To investigate the resistance rate of ryegrass resistant accession, an experiment was established in a completely randomized design with four replicates including all R and S ryegrass accession

In this experiment, a range of herbicide doses which were higher and lower than recommended dose were used on three accessions. Treatments were included nine herbicide treatment rates including an untreated control (0.25, 0.5,1, 2, 4, 8, 16, 32 times the discriminating dosages), which were equal to 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 ppm ai (Zand et al., 2008).Shoot length was measured 7 DAT and was expressed as a percentage relative to control for each biotype.

In order to assess experimental errors the normality test was done prior to analysis of variance, to determine. All data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 2002). Treatment means were compared using Duncan's Multiple Range Test at $P < 0.05$.

To describe the dose–response of ryegrass accessions to clodinafop-propargyl herbicide, to draw dose response curves of herbicide resistant and susceptible populations tested, data using Sigma Plot software were fitted by Logistic Equation 1:

$$Y= C+ (D-C)/1+(x/I_{50})^{b} \quad (1)$$

Where Y is the dependent variable(length of shoot or the number or dry weight of remained plant as a percentage of control without herbicide), X is the herbicide dose, C the minimum curve limit, D, the maximum curve limit, b is the slope of the response curve at ID50 point and ID50 donates the dose producing 50% inhibition of response.

In the case of variable C in the equation 1 equal to zero or was not statistically significant, Logistic Equation 2was used.

$$Y= D/1+(x/I_{50})^{b} \quad (2)$$

Resistance rate of resistant accessions was obtained according to ratio of ED50 of resistant population (R) to ED50 of susceptible population (S) (R/S).

Results and Discussion

Determination of discriminating dose

Results from measuring coleoptile elongation in S accession indicated that dose of 0.01 ppm clodinafop-propargyl caused a 50% inhibition of coleoptile elongation on s accession than control 7 DAT (Figure 1). Analysis of variance of coleoptile elongation confirmed significantly different ($P \leq 0.05$) among three accession from control untreated. No significant different was observed between two R accessions (F1 and F2), whereas, differences was observed in coleoptile elongation between R and S populations. Thus, it can be concluded that coleoptile growth of F1 and F2 was not affected at dose of 0.01 ppm a.i clodinafop-propargyl (dose required for 50% inhibition of shoot growth in susceptible accession), indicating resistance in ryegrass accession to clodinafop-propargyl herbicide.

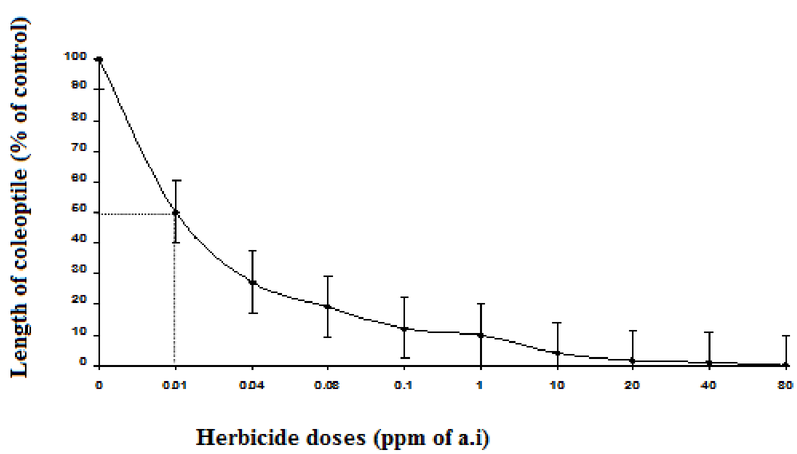

Fig. 1- Effect of different concentrations of clodinafop-propargil herbicide on the length of coleoptile of susceptible biotype (S), as a percentage of untreated controls.

Determine the degree of resistance

Based on the results, resistant biotypes were remarkably different from susceptible biotype in response to concentrations of clodinafop-propargil herbicide 7 DAT (Figure 2). Results are often based on fitting data to logistic model (Equation 1). So that, 0.01 ppm of clodinafop-propargyl, induced inhibition of coleoptile elongation at the rate of 50% in S population compared to control, in contrast, when clodinafop-propargyl herbicide was applied at eight times the discriminating dose in the F1 and F2 biotypes were inhibited by 35 and 15 % , respectively(Fig 2). Also, steep decline curve was higher in S biotype than R biotypes, indicating with increasing herbicide doses, the percent reduction in a coleoptile elongation in S biotype was more sever compared to R biotypes (Table 1).

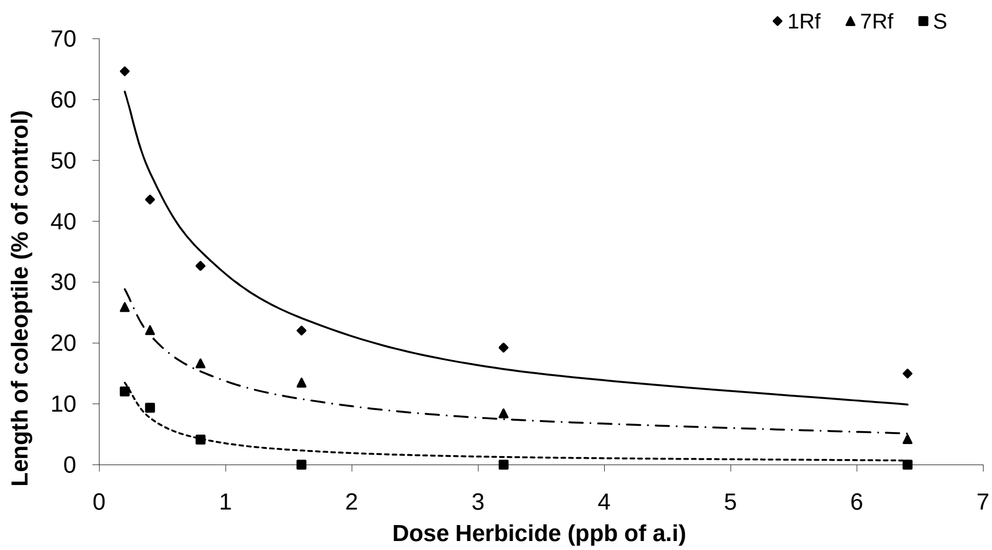

Figure. 2- Effect of different concentrations of clodinafop-propargyl herbicide on length of coleoptile of susceptible (S) and resistant (FR1, FR7) biotypes as a percentage of untreated controls, 7 days after application of clodinafop-propargyl. Symbols and lines represent actual and estimated response of resistant and susceptible biotypes, respectively

Table 1- Parameter estimates obtained for length of coleoptile of susceptible and resistant biotypes as a percentage of untreated controls, 7 days after application of clodinafop-propargyl. The model fitted was Y= D/1+(x/I$_{50}$)^{b} D = lower asymptote, is the slope of the response curve R2 = coefficient of determination.

Biotype	D	b	R2	GR50	R/S
FR1	101.5767	0.7640	0.9883	0.3468	13.5468
FR7	100.2446	0.5807	0.9899	0.0420	1.64063
S	99.9525	0.9041	0.9977	0.0256	

[a]GR50 values are the dosages in g ha-l of herbicide that reduced shoot dry weights by 50% relative to the untreated control 7 d after spraying; [b]R/S is the GR50 ratio of resistant populations to the susceptible

Dose-response experiments

Percent dry weight compared to control

Results of the fitting data to model described by Ritz and Streibig (2005) indicated that there was significance difference between Percent dry weight in S biotype and R biotypes (F1 and F7) compared with control 4 weeks after treatment (Figure 3). As, with increasing herbicide dose, percent dry weight in S population unlike the resistant biotypes was suddenly dropped. For example, in R biotypes, even using the highest dose used of clodinafop-propargyl herbicide (1024 g ai ha-1) which equals to 16 times the recommended dose (64 g ai ha-1), percent dry weight in F1 and F7 biotypes was decreased 49.26 and 69.28 % respectively, compared to control. However, dry weight of S biotype treated to 32 g ai ha-1 (half of the recommended dose), was reduced 82.07 compared with control. Therefore, it can be assumed that F1 population showed the most resistant to clodinafop-propargyl herbicide (with ratio R/S 2299. 9276), followed by F7 population (with ratio R/S 299. 6332) (Table 2).

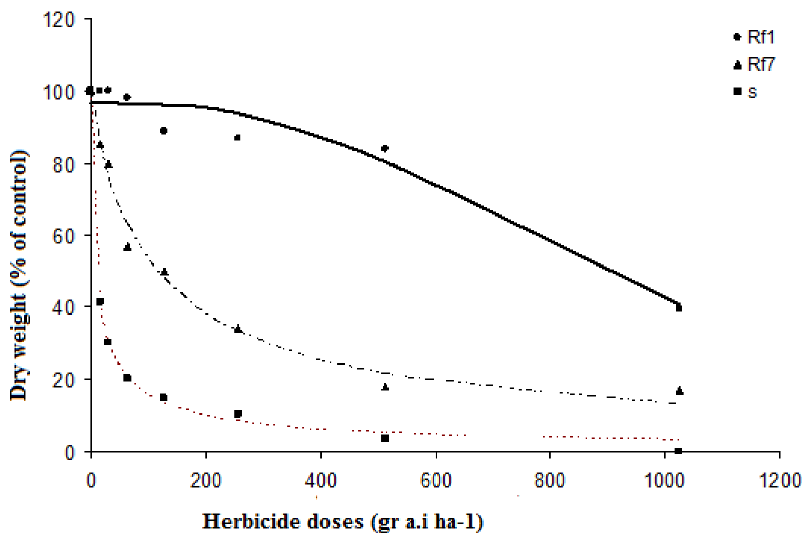

Figure 3- Effect of different concentrations of clodinafop-propargyl herbicide on shoot dry weight of susceptible (S) and resistant (FR1, FR7) biotypes, as a percentage of untreated controls. Symbols and lines represent actual and estimated response of resistant and susceptible biotypes, respectively.

Table 2- Parameters estimates obtained for shoot dry weight of susceptible and resistant biotypes as a percentage of untreated controls, 4 week after spraying clodinafop- propargyl

Biotype	D	b	R2	GR50	R/S
FR1	101.2236	0.8293	0.9838	1030.3676	2299.9276
FR7	93.9575	1.0091	0.9787	134.2375	299.6332
S	99.9675	0.3562	0.9933	0.4480	

Survival plants number (% of untreated control)

Ryegrass accessions showed different performance in response to increasing dose of clodinafop-propargyl herbicide in term of the number of live plants, as a percentage of untreated controls. Based on the fitted models for the number of remaining ryegrass before spraying (Fig. 4) and the R / S ratio (Ritz and Streibig, 2005) (Table 3), F1 population was more resistant than F7 like percent dry weight. For example, at dose of 256 g ai ha-1 which equals to four times the recommend dose, the number of plants live in F1 and F7 populations was found 93.74 and 33.45%, respectively. While, the number of plants live in S population at the recommended dose was lower than 10%.

Overall, the results of dose response curves of accessions for two measured traits (percent dry weight and the percent of live plants number to control), indicated that the degree of resistance was F1>F7>S. Thus, resistant populations can be controlled using doses higher than recommended doses.

R/S factor in resistant accessions for two evaluated traits was greater than 1 (R/S >1) which was statistically significant, confirming resistance of these populations to tested herbicide.

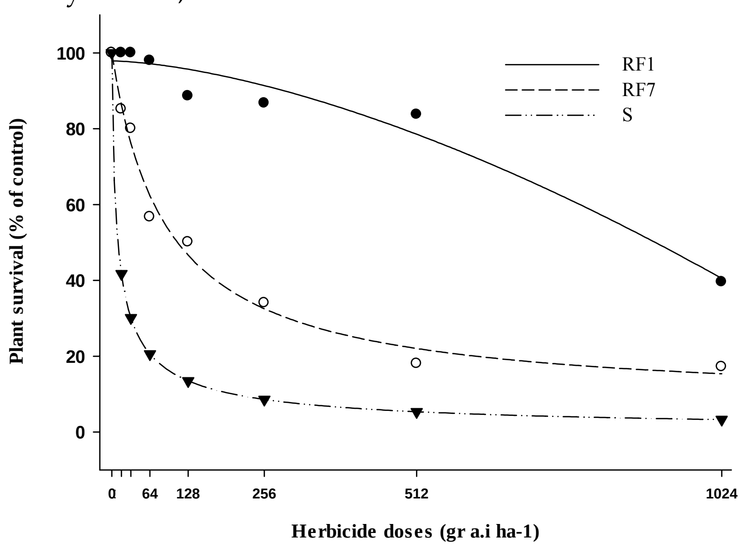

Figure. 4- Effect of different concentrations of clodinafop-propargyl herbicide on survival of susceptible (S) and resistant (FR1, FR7) biotypes, as a percentage of untreated controls. Symbols and lines represent actual and estimated response of resistant and susceptible biotypes, respectively.

Table 3- Parameter estimates obtained for survival of susceptible and resistant biotypes as a percentage of untreated controls, 4 week after spraying clodinafop- propargyl

Biotypes	D	b	R2	GR50	R/S
FR1	96.5385	2.7639	0.9725	912.0332	86.285
FR7	100.7194	0.8634	0.9932	113.9688	10.7822
S	99.9504	0.7334	0.9987	10.570	

Table 4. Mean comparison of percentage injury based on EWRC, the number of survive plant (%) after and before spraying, dry weight (%) at recommended dose (64 g ai ha-1) in Fars Province.

Populations	The percentage of injury (EWRC)	The number of ryegrass after treatment as a percentage of untreated control	Dry weight of ryegrass as a percentage of untreated control
RF1	10.0 c	92.33 d	94.42 a
RF7	55.25 b	68.54 c	59.35 b
RS	96.45 a	25.68 a	6.88 c

Letter differences across treatment means (columns) indicate difference at P, 0.05.

Table 5. Comparison of results resistant accessions using, the number of survive ryegrass as a percentage of control (NO), dry weight as a percentage of control (DM) AND visual assessment in dose response assay.

Indices	Resistance in different populations
NO	RF1>RF7>S
DM	RF1>RF7>S
EWRC	RF1>RF7>S

F1 population having had R/S ratio of 2299.9267 and 86.285 for percent dry weight and the number of plant live, respectively as a percentage of the untreated controls was detected as a the most resistant accession . While, a somewhat intermediate resistance was existed to Clodinafop-propargyl in F7 population of ryegrass population (R/S ratio of 299.6332 and 10.7822, respectively, for mentioned traits). Finally, the two resistant accessions of ryegrass showed I50 greater of 50% and the average of visual assessment more than 6 when exposed to 64 g a.i ha^{-1} of Clodinafop-propargyl, indicating the presence of resistance in these accessions. The slope of the response curve at ID50 point, representing the sensitivity of studied populations of ryegrass to difference doses of herbicide or the speed of response-trend to difference doses of herbicide, in R populations was lower than susceptible population. Furthermore, the descent speed curve in susceptible population was more compared to others. In other words, the distance between maximum and minimum of response was occurred faster in susceptible population of ryegrass. The astonishing point in these results is that when the resistance of different accessions was calculated through the fitted models to the number of plant live and dry weight, the rating of populations is different, while, in this study rating was similar. Currently, to solve this problem and more reliable results of screening experiments, Moss et al. (2007) are suggested that instead of visually rating system, the percent reduction of plant number and the percent reduction of dry weight, was used the reduction of wet weight as a percentage of control.

Obviously, based on two evaluated traits (the number and dry weight of plants), resistance was approved in two accessions of ryegrass identified as a resistant population at screening test. Banakashani et al. (2006) were investigated the suspected resistant wild oat accessions through screening test and dose-response assay. They confirmed that accessions were resistant in dose-response assay; they had been identified as resistant accessions as well.

Comparison of mean score (3 weeks after spraying) indicated that RF1 and S accessions with scores 10 and 96.45 (% of damaged), respectively, Showed the least and maximum damage (Table 4). In screening guidelines expressed that if dry weight and or plant survival (as a percentage of control) is higher than 50%, is a sign of possible resistance (Beckie et al., 2000). According to the results of the analysis and mean comparison of three traits listed, it can be concluded that RF1 and RF7 is resistant to clodinafop-propargyl and S population is susceptible to herbicide listed.

Conclusions

According to the results obtained in this study, resistance in ryegrass populations in the Fars province is determined. However, the degree of resistance among the populations was different greenhouse in and Petri dish experiments, consistent results showed that in vitro methods can be used instead of pot experiments, which not only have the ability to distinguish between biotypes, but also it is applicable at less time and space. Consequently to prevent the distribution of resistant populations across this province, applying the appropriate management practices is essential. Based on the study of De Prado et al.(2000), the resistant biotype of ryegrass was 10-foldmore resistant to diclofop-methyl compared with the susceptible biotype. The levels of resistance in the *Alopecurus myosuroides* and *Lolium multiflorum* to fenoxaprop-ethyl and Clodinafop-propargyl have been determined 22.8 and 41.5 respectively (Tal et al., 2000). The occurrence of cross-resistance in a population of *Lolium rigidum* resistant to aryloxyphenoxypropionate herbicides has been reported by Heap and Knight. (1986). De Prado et al. (2000), also, have confirmed cross-resistance in *Lolium multiflorum* biotype to ACCase inhibitor herbicides.

In this study, evaluation of resistant accessions through various indices, including the number of survival ryegrass (% of control) (NO), dry weight (% of control) (DM) and visual assessment of damage caused by the herbicide treatment compared to control in dose-response experiment (EWRC) (Table 5). Some researchers such as Moss et al (2007) believe that measurement of fresh weight loss than control is a more accurate factor.

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