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Clodinafop-Propargyl Resistance Genes in *Lolium rigidum* Guad. Populations Are Associated with Fitness Costs

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Abstract: Amino acid substitutions that confer herbicide-resistance may cause fitness costs in mutant plants at unfavorable levels in contrast to wild-species. The fitness costs in three *Lolium rigidum* populations (AH3 (Ile-2041-Asn) and BO2 (Ile-1781-Leu) as resistant (R) to clodinafop-propargyl, an ACCase (acetyl-CoAcarboxylase) inhibitor, carrying the mutations 1781 and 2041, respectively, and HF as susceptible (S)) were studied during 2014 and 2016. The germination rates and percentages of the three *L. rigidum* populations, and competition between them and *Triticum aestivum* using substitution series experiments were assessed. The BO2 and AH3 populations showed resistance to clodinafop-propargyl due to mutations in their ACCase genes. The germination rate for *L. rigidum* decreased as the sowing depth increased, with the lowest germination rate being found at 8 cm. AH3 and HF populations presented higher seed germination under water and NaCl salinity stress, but no fitness cost variations were observed among these R populations under optimal growth conditions. Diverse germination responses to light conditions were observed between the S and R *L. rigidum* populations. The highest germination percentage was observed in the HF population at the two-week lighting + two-week darkness regime. The comparison of relative yield total and relative crowding coefficient showed that *T. aestivum* was more competitive than *L. rigidum*. However, among ACCase-resistant *L. rigidum* populations, AH3 population was the most competitive presenting no fitness costs. This R population was more competitive than the S (HF) one under competitive conditions. These results show that fitness costs in the R *L. rigidum* populations vary according to the specific mutation at the ACCase gene that confers resistance to clodinafop-propargyl. In conclusion, mutations occurring at the 2041 position in the ACCase gene caused fitness costs, but those occurring at the 1781 position did not generate fitness costs for *L. rigidum*. Therefore, non-chemical methods should be considered unfavorable for resistant populations of this species.

Keywords: fitness cost; germination; light regime; mutation; salinity stress; water stress

1. Introduction

ACCase (acetyl-CoAcarboxylase)-inhibitors, introduced in 1975 [1], are selective herbicides used to control grass weed species [2]. These herbicides do not affect dicotyledonous plants, and some monocotyledonous crops, such as wheat (*Triticum aestivum* L), are naturally tolerant to them [3]. The chemical families of ACCase-inhibitors are: hydroxyphenoxyisopropionic acid (AOPP), hydroxyoxocyclo-hexenecarbaldehyde oxime (CHD), and phenyloxopyrazolinyl formate (PPZ) chemical families [4–6]. They act on the ACCase, a nuclear encoded enzyme located in the chloroplasts of plants [7–9].

Lolium rigidum Guad. (Rigid ryegrass) is widely distributed throughout many countries [10,11]. It is the dominant weed in Iranian crop production systems, and is considered one of the most difficult species to control for grain production in most areas of the country [12]. Resistance to ACCase inhibitors was first reported in 1982 [8]. To date, *L. rigidum* has been reported as having evolved resistance to 11 herbicide modes of action in 12 countries [13], showing the wide-spread incidence of herbicide resistant *Lolium* spp. [13,14].

The mechanisms involved in herbicide resistance are [15,16] TS (target-site) and NTS (non-target-site) resistance in nature. TS-resistance is often reported in ACCase inhibitor-resistant weeds, and is endowed by modifications in the target site of ACCase inhibitors [17]. NTS-resistance mechanisms are related to a decrease in the herbicide uptake and/or translocation, or an increased herbicide detoxification [15,18,19].

Resistance mechanisms for various mode of action (MOA), such as ACCase [20], 5-enolpyruvylshikimate-3-phosphate synthase [21], and photosystem II [22], inhibitors may also represent fitness costs in different weed species [22–24]. The fitness costs are dependent on population genetic backgrounds, resistance mechanisms (TSR or NTSR) involved, and the field conditions [9]. Various mutations can influence the fitness cost of weeds [25]. The Ile-2041-Asn replacement in the ACCase gene, for example, decreased the total mass and seed production in *Hordeum glaucum*, while the Ile-1781-Leu/Val replacement did not generate fitness costs [26].

ACCase-resistant *L. rigidum* populations have been identified in winter cereal fields in Iran [24]. The resistant (R) populations evaluated in this study presented a high resistance level to clodinafop-propargyl, an ACCase-inhibitor herbicide. The objective was to evaluate the fitness cost of the resistance to this herbicide in two R *L. rigidum* populations (AH3 and BO2, carrying mutations at the 1781 and 2041 positions, respectively, responsible for resistance to ACCase-inhibitor herbicides), compared with a susceptible (S) population (HF). The potential fitness cost was studied in terms of rate and percentage of germination, water and NaCl salinity stresses, and the effect of light regimes on seed germination of AH3, BO2 and HF populations.

2. Materials and Methods

2.1. Plant Materials

An HF (susceptible) population, used as control, was collected from a field with no history of herbicide application. AH3 (Ile-2041-Asn) and BO2 (Ile-1781-Leu) resistant *L. rigidum* [24], which survived repeated clodinafop-propargyl (Topik®, Syngenta, Basle, switzerland) applications, were collected from winter fields in Iran in 2013–2014. Spikes and seeds were stored at temperatures between 20 and 25 °C until testing. Seeds were sown in 12 × 12 × 10 cm plastic pots containing a manure-loam-sand mix (40:40:20% *v*/*v*/*v*), and watered twice a week. Seedlings were kept in a greenhouse at 21–26/11–16 °C day/night, with a 16-h photoperiod and 50–60% relative humidity.

2.2. Greenhouse Dose-Response

Lolium rigidum plants from two selected R biotypes and the S biotype, at the 3–4 leaf stage, were sprayed with the following clodinafop-propargyl doses: 0, 16, 32, 64, 128, 256, 512, and 1024 g ai ha^{-1}. Herbicide application were performed using an backpack sprayer (Marolex, Warsaw, Poland)

equipped with a 11,002 Tee-Jet nozzle (Keshtpoosh, Tehran, Iran) spaced 50 cm apart, and calibrated at 200 kPa to deliver 250 L of herbicide mix ha^{-1}.

Lolium rigidum control was visually determined at 14 and 28 days after treatment (DAT) determining a percentage of 0 to 100% (0% = no injury and 100% = plant death). The control degree was based on signs, such as individuals with chlorosis and necrosis, compared to untreated individuals. Above-ground biomass was harvested at 28 DAT from each test, dried at 72 °C and weighed. The biomass data was contrasted with control plants.

2.3. Effect of Burial Depth on Germination Rate (GR) of AH3, BO2, and HF Populations

In the burial depth experiment, the effect of planting depth on germination rate in the HF, BO2, and AH3 population was measured. S and R seed populations were planted in 500 mL volume pots at predetermined depths (0–8 cm). Seedlings were put in a greenhouse under controlled conditions at 21–26/11–16 °C day/night, 16-h photoperiod, 50–60% relative humidity and the pots were irrigated at specific times. The test was conducted in a completely randomized design (CRD) with four replications. Seed germination was continued for three weeks.

2.4. Water Stress and NaCl Salinity Stress on Germination Percentage (GP) of AH3, BO2, and HF Populations

Seeds of BO2, AH3, and HF populations were sterilized in 1% sodium hypochlorite solution for 2–3 min, rinsed with purified water, and air-dried for 4 h before being placed in Petri dishes. Treatments included polyethylene glycol 6000 (0, 3, 6, 9, 12, and 15 bar) and NaCl (0, 5, 10, 15, 20, and 25 decisiemens per centimeter (ds cm^{-1})). Twenty seeds from each population were put on two filter paper disks in 9-cm Petri dishes with an 8.0 cc prepared dilution (for PEG 6000 and NaCl) and distilled water (for the rest of treatments). The germination percentage was recorded daily for two weeks. Seeds were considered germinated when the radical presented a length of at least 0.2 cm [20].

2.5. Effect of Light Regimes on Seed Germination of AH3, BOS2, and HF Populations

Seeds from each *L. rigidum* population were tested immediately. Spikes from the wheat fields were harvested and stored at a temperature between 21 and 26 °C. The germination percentage was assayed at two times during the following year. Twenty seeds from each population were incubated at 21/11°C on two filter paper disk in 9-cm Petri dishes under three light regimes: two weeks with 12 h light (14 dL); two weeks in darkness (14 dD) or two weeks in darkness followed by two weeks exposure to 12 h lights (14 dD + 14 dL). Light was blocked by covering the sheet in aluminum foil.

2.6. Comparison of Relative Yield Total (RYT) and Relative Crowding Coefficient (RCC) between T. aestivum and L. rigidum

These experiments were conducted to measure unfavorable effects, growth and seed production of AH3, BO2, and HF *L. rigidum* populations and *T. aestivum* in competitive and non-competitive situations. For this purpose, pots were put in controlled greenhouse situations at 21–26/11–16 °C day/night, 16-h photoperiod and 50–60% relative humidity. The pots were irrigated at set times. Pots were filled with manure-loam-sand mix (40:40:20% $v/v/v$). Competition between *L. rigidum* populations and *T. aestivum* was essayed by methods of a substitution series experiment at five *L. rigidum*: *T. aestivum* densities (8:0, 6:2, 4:4, 2:6 and 0:8).

RYT and RCC were used to determine competitive ability and as an indication of the competitive ability of a crop to obtain limited solar, nutritional and water resources when grown together with weed. This was contrasted with the ability of plants to use those resources when grown in a pure stand [23]. A RYT or RCC > 1.0 signifies a competitive benefit for *L. rigidum* (S and R populations) when compared with *T. aestivum*, whereas, an RYT or RCC of 1.0 indicates that *L. rigidum* and *T. aestivum* species are making demands on the same resources. If RYT or RCC is less than 1.0, this indicates antagonism between *L. rigidum* and *T. aestivum* plants [27].

2.7. Data Analysis

The dose to reduce the dry weight (GR_{50}) and to kill plants (LD_{50}) by 50% was specified using the 3 parameter log-logistic curve $Y = d/1 + \exp [b (\log(x) - \log(e))]$ [1]; where the biomass reduction is Y, the upper limit is d and the lower limit is c, e represents the GR_{50} value, b is the relative slope around the parameter e, and x is the herbicide dose (g ai ha^{-1}). Resistance indices (RI = R/S) were calculated as the ratio of R to S GR_{50} values.

The germination percentage was calculated using the equation: PG = ni/$N \times 100$ [28]. The germination percentage (PG) is the total number of germinated seeds (ni) divided by the total number of seeds (N). RYT is the total RY of the two associated plants: RYT = (Ytl/Ytt) + (Ylt/Yll) = $RY_1 + RY_t$. Relative yield (RY) is the ratio of the species response (shoot dry weight) in the mixture and the plant response when grown in a monoculture. The RCC for dry weight production for *L. rigidum* and *T. aestivum* was calculated as: RCC = RCClt $\times$ RCCtl; where RCClt = YltXtl/(Yll − Ylt)Xlt; RCCtl = YtlXlt/(Ytt − Ytl)Xtl; Ylt and Ytl indicates the dry weight of *L. rigidum* and *T. aestivum*, respectively; Yll and Ytt represent the dry weight in pure cultures of A and B species; and Xtl and Xlt are the ratios for planting for A and B species.

3. Results

3.1. Herbicide Screening Experiment

Different resistance levels to clodinafop-propargyl were identified between the resistant *L. rigidum* populations at 30 days after spraying. AH3 population was the most resistant based on survival and fresh-weight. The BO2 population also had a high resistance rate to clodinafop-propargyl. Based on the screening test, the AH3 and BO2 populations were classified as resistant to clodinafop-propargyl, and these populations were selected for dose-response test.

3.2. Dose–Response Assay in the Greenhouse

All HF (S) individuals died in the treatment with 32 g ai ha^{-1} of clodinafop-propargyl (50% of the recommended rate), while the AH3 and BO2 populations exhibited high survival rates. Based on GR_{50} and LD_{50} values of the HF population (5.8 and 6.9 g.ai ha^{-1}, respectively), the resistance index of the resistant *L. rigidum* populations ranged from 20.4 to 316.3 in terms of the fresh weight reduction, and from 29.1 to 174.2 for the survival percentage (Table 1).

Table 1. Clodinafop-propargyl doses (g ai ha^{-1}) needed to reduce the fresh weight (GR_{50}) and survival percentage (LD_{50}) by 50% in *Lolium rigidum* biotypes from Iran.

Population	Fresh Weight				Survival			
	d	***b***	**GR_{50}**	**R/S**	***d***	***b***	**LD_{50}**	**R/S**
AH3	99.5	1.6	1866.4	316.3	101.4	0.7	905.6	174.2
BO2	100.0	1.0	120.3	20.4	101.5	0.9	151.2	29.1
HF (S)	100.0	1.6	5.9		100.0	1.6	5.2	

RI = Resistance index (R/S) calculated using the corresponding GR_{50} or LD_{50} values of the resistant biotype with respect to the susceptible one. *d*: the upper limit, *b*: is the relative slope around the parameter *e* (*e* represents the GR_{50} value). GR_{50}: the mean dose that caused growth reduction by 50%; LD_{50}: the mean dose that caused mortality reduction by 50%. AH3, BO2, HF: the biotypes collected from the Ahvaz, Bostan and Haftkel counties respectively.

Comparing the ACCase-gene fragments of the *L. rigidum* populations, the BO2 population was the only one that revealed a single nucleotide mutation from ATT to CTT at the 1781 location (Ile-1781-Leu). The AH3 population revealed the single nucleotide mutation from ATT to AAT at the 2041 location (Ile-2041-Asn).

3.3. Effect of Burial Depth on Germination Rate (GR) of R and S Populations

The decreasing rate of germination (3.4) was more evident in the HF population than the resistant ones, while the slope for AH3 was 2.9 while for the BO2 resistant mass it was 2.8. The three populations showed a decrease in the germination rate with increasing depth. On the soil surface (0 cm), the highest GR was observed in the S population and at the final depth (8 cm). The germination rate in the BO2 and AH3 populations was similar, but the HF population showed a decrease in comparison with the other populations. In general, with increasing depth, the maximum germination rate decreased, but the decrease in the S population was lower than that of resistant populations (Figure 1).

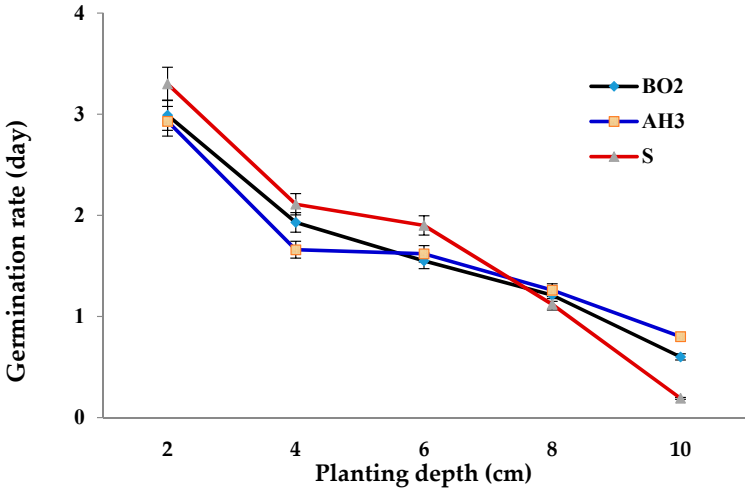

Figure 1. Effects of sowing depth on germination rate (GR) in HF (S) (red bars), BO2 (Ile-1781-Leu) (black bars), and AH3 (Ile-2041-Asn) (blue bars) *Lolium rigidum* populations. The corresponding equations are: Population S: y = 3.3371 + 0.2006 x, (r^2 = 0.92); Population BO2 (Ile-1781-Leu): y = 2.7655 + 0.1520 x, (r^2 = 0.91); Population AH3 (Ile-2041-Asn): y = 2.9417 + 0.1724 x, (r^2 = 0.97).

3.4. Water Stress and NaCl Salinity Stress on Germination Percentage (GP) of R and S Populations

Seed germination was different for the AH3, BO2, and HF populations and diminished due to a decrease in osmotic potential affected by polyethylene glycol from −3 to −12, while in −15 bar it reached its lowest level (Figure 2). Seed germination percentages of the AH3 and BO2 populations were clearly lower in comparison with the HF population. Coefficients from the linear regression equation of seed germination resistant and susceptible biotype data showed that the BO2 biotype, compared to AH3 and HF, was more tolerant of water stress, and in water potential 7.5, its seed germination was reduced to 50% (Figure 2). The seed germination percentages in the AH3, BO2, and HF populations were 92.5, 93.8, and 98.8 in the control treatment, respectively. The concentration of salinity at which 50% of HF and BO2 seeds reached germination is greater than AH3. Hence, the HF and BO2 concentrations of 21 dS cm^{-1}, and the AH3 biotype at a lower level of salinity (19 dS cm^{-1}) reach 50% germination (Figure 3). Similar to the results for the water stress test, a significant population effect on the seed germination was seen under NaCl salinity stress; AH3 and BOS2 population levels were clearly lower in comparison to the HF population (Figure 3).

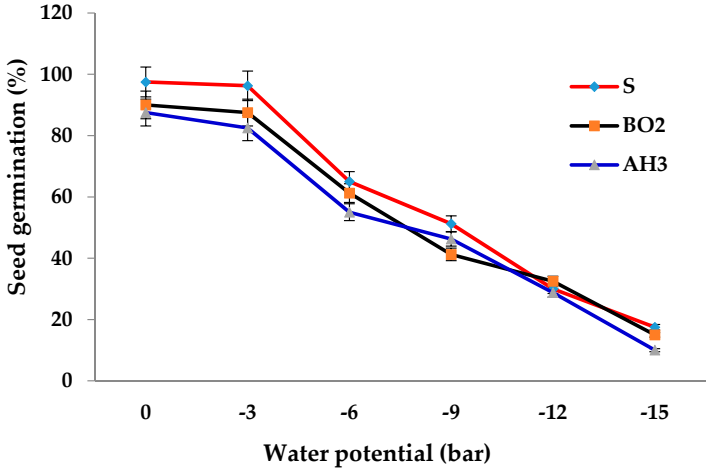

Figure 2. Final germination of the HF (S) (red bars), BO2 (Ile-1781-Leu) (black bars), and AH3 (Ile-2041-Asn) (blue bars) *Lolium rigidum* populations under water stress. The corresponding equations are: population S: $y = -17.404 + 0.166x$, std. error: 0.983, ($r^2 = 0.97$); population BO2 (Ile-1781-Leu): $y = -17.436 + 0.182x$, Std.Error: 0.966, ($r^2 = 0.97$); population R2 (Ile-2041-Asn): $y = -17.024 + 0.184x$, std. error: 0.795, ($r^2 = 0.98$).

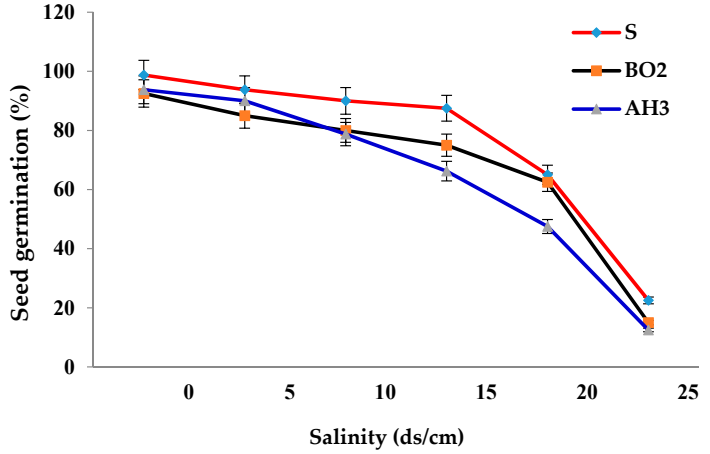

Figure 3. Final germination of the HF (S) (red bars), BO2 (Ile-1781-Leu) (black bars), and AH3 (Ile-2041-Asn) (blue bars) *Lolium rigidum* populations under water stress. The corresponding equations are: population S: $y = 34.121 - 0.284x$, std. error: 6.398, ($r^2 = 0.77$); population BO2 (Ile-1781-Leu): $y = 32.543 - 0.293x$, std. error: 5.832, ($r^2 = 0.78$); population R2 (Ile-2041-Asn): $y = 31.308 + 0.290x$, std. error: 3.298, ($r^2 = 0.91$).

3.5. Effect of Light Regimes on Seed Germination of R and S Populations

Powerful dormancy was obvious in seeds assayed immediately after collection, with less than 7.5% germination for AH3, BO2, and HF populations (Table 2). Dormancy decreased over time for the three populations, with up to 50% germination in darkness after one year (365 days) of storage at room temperature. When the seeds were placed in light after being in darkness, most germinated. Seed germination of all populations increased significantly after one year of storage, however, seed germination in the BO2 population was less than that in the AH3 and HF populations (Table 2). Imbibitions of seeds of the BO2, AH3, and HF populations in darkness, followed by a light period (14 dD + 14 dL), increased seed germination in contrast with that observed with only darkness or lighting only (14 dD, 14 dL) after the ripening period (Table 2). Of the three populations, the seed germinations in the BO2 population were the lowest in darkness with consequently fewer seedlings

emerging. Therefore, it seems that a cycle of dark imbibitions followed by light accelerated dormancy release in seeds of AH3, BO2 and HF populations.

Table 2. Final germination of the HF (S), BO2 (Ile-1781-Leu) and AH3 (Ile-2041-Asn) populations at diverse after-ripening times at 11/21 °C and under three light treatments: two weeks darkness (14 dD), exposure to 12 h light (14 dL), and dark imbibed for 14 days followed by disposal for 14 days to 12 h light (14 dD + 14 dL).

Light Regimes	Biotype	Day	
		0	365
14 dD	HF (Susceptible)	7.5	62.5
	BO2 (Ile-1781-Leu)	3.0	25.0
	AH3 (Ile-2041-Asn)	5.2	45.0
14 dL	HF (Susceptible)	46.5	90.0
	BO2 (Ile-1781-Leu)	26.5	76.5
	AH3 (Ile-2041-Asn)	40.0	85.5
14 dD + 14 dL	HF (Susceptible)	75.0	97.5
	BO2 (Ile-1781-Leu)	65.0	85.0
	AH3 (Ile-2041-Asn)	67.5	92.5

3.6. Comparison of RYT and RCC of T. aestivum and L. rigidum (S and R Populations)

The RYT of *T. aestivum* and HF, BO2 and AH3 *L. rigidum* populations, in all ratios, was about 1.0 and less than 1.0, indicating an antagonistic effect between *T. aestivum* and these populations. The RCC was higher than 1.0 for dry weight, displaying a low competitive benefit for *T. aestivum*. The indices were calculated using the substitution series study, and provided valuable understanding of the relative competitive abilities of *T. aestivum* with *L. rigidum*. In fact, the highest RCC value was between 0.13 and 2.37, showing the competitive advantage of *T. aestivum* over *L. rigidum*. The RCC indices indicated competition between the BO2, AH3, and HF populations of *L. rigidum* with *T. aestivum*. The AH3 population showed the greatest competitiveness, and the BO2 population was the least competitive with *T. aestivum* (Table 3).

Table 3. Comparison of relative yield total (RYT) and relative crowding coefficient (RCC) based on shoot dry weight of *T. aestivum* and *L. rigidum* (S and R populations) in different ratios of sowing *L. rigidum* and *T. aestivum.*

Populations	Ratio	RYl	RYt	RYT	RCCl t	RCCtl
HF (Susceptible)	25:75	0.16	0.73	0.89	0.65	1.53
	50:50	0.26	0.59	0.85	0.47	2.13
	75:25	0.65	0.30	0.95	0.73	1.37
BO2 (Ile-1781-Leu)	25:75	0.13	0.89	1.02	0.42	2.37
	50:50	0.32	0.65	0.96	0.50	2.01
	75:25	0.63	0.35	0.98	0.66	1.51
AH3 (Ile-2041-Asn)	25:75	0.20	0.77	0.97	0.73	1.37
	50:50	0.41	0.56	0.97	0.71	1.41
	75:25	0.67	0.25	0.95	0.88	1.14

RYr: Relative yield of *L. rigidum*; RYt: relative yield of *T. aestivum*; RYT: relative yield total; RCCrt: relative crowding coefficient of *L. rigidum* to *T. aestivum*; and RCCrt: relative crowding coefficient between *T. aestivum* and *L. rigid.*

4. Discussion

Previous studies on the fitness costs of resistance to ACCase inhibitor herbicides in *L. rigidum* populations showed no distinctions in the competitive capabilities of resistance against the susceptible population [29,30]. The results found in this study indicated that ACCase herbicide resistant *L. rigidum*

showed a considerable difference between the BO2 (with the ACCase 1781-mutation) and HF (S-susceptible) plant populations.

In the screening experiment, two populations were the most resistant biotypes to clodinafop. These populations showed RI ranging from 29.1 to 316.3 times higher compared with the susceptible population. The evolution of resistant individuals in a population results from selection pressures caused by repetitious use of herbicides with the same mode of action [31]. Obligatory cross-pollination weeds, such as *Alopecurus* sp. and *L. rigidum*, help to avoid these resistance traits [17].

Two resistant *L. rigidum* populations were genotyped to identify ACCase target-site resistance mutations. The mutations 1781 and 2041, identified in the BO2 (the first) and AH3 (the second) populations, were found conferring resistance to APP herbicides [32–34], explaining their resistance to clodinofop. In *Lolium* species seven mutations at positions Ile-2041, Cys-2088, Ile-1781, Asp-2078, Trp-1999, Gly-2096, and Trp-2027 have been reported giving resistance to ACCase inhibitors [34,35].

The AH3 population showed higher competitive ability compared with the HF population, in terms of germination rate, seed germination percentage, biomass, and reproductive capabilities. Previous studies in various grass weed species have reported a positive correlation between fitness cost and biomass production [36,37]. In competition with wheat plants, the AH3 population was less affected compared with either BO2 or HF plants. Corroborating previous studies, our experiment with R plants revealed that various mutations can be responsible for the diverse competitive abilities of AH3 plants showing fitness greater than either HF or BO2 plants in various traits [32,38–41].

Seeds from BO2 and AH3 populations showed a fitness cost under NaCl salinity stress and water stress in comparison with the HF population. However, no fitness variation was observed under optimal test conditions. This constant response to low osmotic potential and high NaCl salinity indicated that seeds from the HF population had a greater ability for germination under stressful conditions in comparison with resistant populations (AH3 and BO2). The lack of fitness costs for R populations relative to the S population is in agreement with the findings described in weed species, which indicated no fitness penalty related to the 1781 and 2041 mutations [22,29].

Light conditions were found to be a significant factor that determined the diverse responses between the populations. Non-constant light for two weeks, either with or without pre-imbibitions in the dark was found to be the ideal condition for germination of AH3, BO2 and HF populations, corroborating prior research into germination percentages in *L. rigidum* [29,42]. Under appropriate environmental conditions, there was little difference in seed germination between the AH3, BO2, and HF populations. However, in the lower quality environment (either stable darkness), lower proportion of BO2 individuals germinated in comparison with HF or AH3 individuals. This decreased germination percentage in the BO2 population in the dark lead to a smaller proportion exposed of seedlings being exposed. Even one year after ripening 60% of the BO2 individuals still needed light to germinate. A cycle of imbibitions in darkness led to an increased germination percentage under lighting and generated a greater germination rate of seeds from the three populations, showing that the intermittent dormancy release mechanism is a typical response in *L. rigidum* populations [29] independent of herbicide resistance.

Summarizing, mutations occurring at the 2041 position in the ACCase gene caused fitness costs, but those occurring at the 1781 position did not generate fitness costs for clodinafop-propargyl resistant *L. rigidum* populations collected in winter cereal fields from Iran. Therefore, non-chemical methods should be considered unfavorable for resistant populations of this species.

5. Conclusions

In summary, the fitness in three *L. rigidum* biotypes were studied that two biotypes were resistant to clodinafop-propargyl (two ACCase mutations 1781-Leu and 2041-Asn) and susceptible biotype. Moreover, it was shown that the mutations with heterozygous 2041-Asn and the 1781-Leu confer a sufficient level of resistance to ACCase- inhibiting herbicides. Fitness cost is unavoidable phenomenon related with TSR changes. These results exhibit that diverse replacement in the similar target-gene

would not inevitably have the identical impact on the rate of fitness cost. The resistance to ACCase inhibitors has happened in *L. rigidum* populations in the wheat fields from Iran. Therefore, farmers improve and enhance their weed handling systems or use herbicides with various mechanisms of function and in a sustainable way, resistance to ACCase-inhibitors will further expand and become a main issue in this country in the near future.

Author Contributions: H.S.Z.: project manager; H.R.M.C.: supervisor; E.Z.: supervisor; and I.T., R.D.P., R.A.-d.l.C., and M.T.A.: consultants. All authors wrote and approved the manuscript.

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