

## Acetyl-CoA carboxylase herbicide resistance and fitness of spring milletgrass (*Milium vernale*) populations infesting winter cereal monoculture in Greece

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### Abstract

Herbicide resistance constitutes a production threat in cereals monoculture. Two putative resistant (R1, R2) spring milletgrass (*Milium vernale* M. Bieb.) populations, originating from winter cereal monoculture fields in northwestern Greece, were studied to elucidate the levels and underlying mechanisms of resistance to acetyl-CoA carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting post-emergence herbicides. Whole-plant rate-response assays showed that the R1 and R2 populations were highly cross-resistant to aryloxyphenoxypropionate ACCase-inhibitors clodinafop-propargyl and diclofop-methyl, but they were susceptible to the cyclohexanedione ACCase-inhibitor clethodim and the ALS-inhibitors mesosulfuron-methyl + iodosulfuron methyl-sodium and pyroxsulam. The analysis of the *ACCase* gene sequence revealed a point mutation (ATT to ACT, GTT, GCT) at 2041 position resulting in an amino acid substitution from isoleucine (Ile) to threonine (Thr), valine (Val) or alanine (Ala). By contrast, all sequenced plants of the S population were found with the wild-type allele encoding Ile-2041. The R1 population grown in the absence of competition produced more fresh weight and tillers than the S population, while both R1 and S populations grown in competition with winter wheat or barley produced similar fresh weight and tillers. Winter wheat or barley grown in competition with either R1 or S population produced similar fresh weight, tillers, and ears. Conclusively, two spring milletgrass populations have evolved target-site mediated cross-resistance to aryloxyphenoxypropionate herbicides, but the competitive ability of the R1 was similar to that of S population, suggesting lack of association between ACCase mutation and weed fitness.

**Keywords:** ACCase inhibitors; growth rate; weed-crop competition; weed resistance

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## Introduction

Spring milletgrass (*Milium vernale* M. Bieb.), known also as early millet, is a self-compatible, autogamous (self-fertilizing), winter annual grass weed of the Poaceae family. It is native to southern Europe and western Asia (Skoglund, 2007; Sheidai and Moghaddam, 2009; Pacanoski and Mehmeti, 2019), while in North America occurs in Idaho where it infests winter wheat crops and the surrounding pastures (Barkworth *et al.*, 2007). It prefers light-textured soils that remain moist during winter months and its seeds are easily transported by wind and can also disperse as a contaminant in harvested cereal grains (Tutin *et al.*, 1980). Heavy infestations of spring milletgrass occur almost exclusively in northwestern Greece, especially where winter cereal monoculture is practiced (Damanakis, 1983). Although less competitive than sterile oat (*Avena sterilis* L.), ryegrasses (*Lolium* spp.), or bromes (*Bromus* spp.) grasses, it engenders considerable crop yield reductions when it occurs at high densities (Damanakis, 1978).

The increased presence of grass weeds in arable crops during the recent years has resulted from the changes implemented to agricultural production systems, such as adoption of short rotations, increased-repeated cultivation of winter cereals, reduced/minimum/no-tillage practices, and inevitable heavy dependence on chemical control management (Massa *et al.*, 2013). In Europe, the range of herbicides and modes of action registered for selective postemergence grass weed control in cereal crops is rather limited. More specifically, chlorsulfuron applied either pre- or postemergence, mesosulfuron methyl + iodosulfuron-methyl-sodium and pyroxulam applied postemergence, and the foliar applied acetyl-CoA carboxylase (ACCase)-inhibitors clodinafop propargyl, diclofop-methyl, fenoxaprop, and pinoxaden are employed to target and effectively manage grass weed infestations. The intensive and recurrent use of ACCase-inhibitors for grass weed control has resulted in the evolution of resistance in 51 grass weed species (Heap, 2024). Currently, weed resistance to herbicides is widespread and affects large cultivated areas of arable crops and particularly winter cereals (Délye *et al.*, 2013; Heap, 2024).

The ACCase herbicide resistance in grass weeds generally involves two resistance-endowing mechanisms, target-site (TSR) and non-target-site (NTSR) resistance. The TSR mechanism is controlled by a single dominant or semi-dominant nuclear gene and is conferred by either increased expression of the target protein or by spontaneous mutations, resulting in single amino acid substitutions at the carboxyltransferase (CT) domain of the plastidic ACCase found in grass weed species. These structural changes of the herbicide target protein reduce effective binding of some ACCase inhibitors and generally confer high resistance levels at the whole plant level (Kaundun, 2014). NTSR is multigenic and driven by complex evolutionary pressures that induce diverse resistance mechanisms, such as enhanced metabolism, reduced uptake, impaired translocation, and herbicide detoxification of the active ingredient into a non-toxic molecule due to oxidation, hydrolysis, or reduction (Yu and Powles, 2014). NTSR is favored by a selection of minor genes when weeds are constantly exposed to sublethal herbicide rates resulting in the accumulation of adaptation genes (Kaundun, 2014). This mechanism is also responsible for unpredictable patterns of herbicide resistance, not specific to the mode of action of the selecting herbicide(s) and differs among and within species (Délye *et al.*, 2013; Yu and Powles, 2014). Regarding the NTSR mechanism of the ACCase-inhibiting herbicides, elevated levels of cytochrome P450 enzymes (Iwakami *et al.*, 2019) or glutathione S-transferases and O-glycosyl transferases usually confer it (Cummins *et al.*, 2013; Kaundun, 2014). This type of resistance is very common to both, acetyl-CoA carboxylase and acetolactate-synthase inhibitors in grass weeds (Délye *et al.*, 2013). Generally, the herbicide resistant patterns determined in most cases of field-evolved resistance to ACCase inhibitors usually reflect herbicide use patterns, selection pressure imposed, and plant fitness associated with specific mutations (Vila-Aiub *et al.*, 2009).

Fitness is defined as the ability of an individual to successfully establish, survive, and reproduce in a given environment, and affects greatly the spread and establishment of herbicide resistance endowing mutations in crop fields (Menchari *et al.*, 2008). Determining changes in fitness associated with the field-evolved herbicide

resistance trait is very important for predicting resistance dynamics and designing the implementation of effective management strategies. In addition, elucidating possible pleiotropic effects associated with herbicide resistance-conferring mutant alleles is indispensable for assessing fitness cost or advantage of a weed grown in its environment, since the pleiotropic effects are influenced by the weed species and the resistance-endowing gene or mutant allele, the genetic background, and the prevailing environmental conditions. Furthermore, pleiotropic effects due to weed resistance are associated with considerable growth rate and competitive ability changes (Vila-Aiub *et al.*, 2009; Délye *et al.*, 2013).

The present study was initiated when growers in winter cereal growing areas of northwestern Greece complained for unsatisfactory control of some spring milletgrass populations after postemergence application of ACCase-inhibiting herbicides. Therefore, as previous research has not investigated the possible resistance evolution and competitiveness of these populations, a study was conducted in order to examine whether this unsatisfactory control was due to the evolution of resistance to ACCase- or ALS-inhibiting herbicides, or the possible field-selection of multiple resistance to both herbicide modes of action. The specific objectives of this work were: i) to study whether the reported unsatisfactory control of spring milletgrass populations claimed by cereal farmers in northwestern Greece was due to the evolution of resistance to some registered ACCase-inhibiting herbicides, ii) to examine the resistant profile of the putative resistant spring milletgrass populations (evolution of cross- or multiple resistance to ACCase- or/and ALS-inhibiting herbicides), iii) to elucidate the possible target-site mediated resistance mechanism, iv) to study the growth rate of one herbicide resistant (R) and one susceptible (S) spring milletgrass populations in the absence of crop competition, and v) to compare the competitive ability of R and S spring milletgrass populations against winter wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.).

## Materials and Methods

### *Seed collection and source populations*

In the early summer (before harvest) of 2022, mature seeds of spring milletgrass were collected from plants that survived management inputs in two monoculture winter cereal fields located in northwestern Greece, where reduced susceptibility of spring milletgrass was experienced after the application of the ACCase-inhibitors clodinafop-propargyl and diclofop-methyl. The fields were located in the prefecture of Grevena (R1; 40° 10437 N, 21° 59792 E and R2; 40° 09364 N, 21° 59411 E). In each field, seeds were collected manually from individual plants grown in 12 different patches in each field, pooled as bulk samples (one single bulk from each field was created), and treated as distinct populations. Additionally, mature seeds were collected from a spring milletgrass population at the margins of a field located right next to the field where the R1 population was collected (S; 40° 10418 N, 21° 59781 E). In this field, spring milletgrass was successfully controlled and was considered as S (reference) population. The collected spring milletgrass seeds were initially placed in large plastic bags, then transferred to the laboratory, air-dried, placed in paper bags, and stored at room temperature for at least two months to allow after-ripening. The seeds were later used in the subsequent experiments.

### *Whole-plant rate-response assays*

Whole-plant rate-response pot experiments were conducted and repeated (experimental runs) at different times at the University Farm of Florina during the autumn of 2022, where the two suspected ACCase herbicide-resistant spring milletgrass populations (R1 and R2) were evaluated for potential ACCase-inhibitor cross-resistance or multiple resistance to ALS-inhibitors. The considered S population was also included in the study. Each 0.9 L plastic pot filled with a 1:1 peat-sand mixture was planted with approximately 35 spring milletgrass seeds, which were placed at a depth of approximately 1 cm. Once seedlings reached the two-leaf stage, they were thinned to six per pot. The putative R1 and R2 populations were treated with four rates [1× (recommended), 2×, 4×, 8×] of the ACCase-inhibitors clodinafop-propargyl (Sword<sup>®</sup>, 240 g ai L<sup>-1</sup>, K&N

Efthymiadis), diclofop-methyl (Keylofop<sup>®</sup>, 360 g ai L<sup>-1</sup>, Farma-Chem), and clethodim (Vetri<sup>®</sup>, 240 g ai L<sup>-1</sup>, K&N Efthymiadis) as well as of the ALS-inhibitors mesosulfuron-methyl + iodosulfuron-methyl-sodium (Atlantis<sup>®</sup>, 29 + 5.6 g ai kg<sup>-1</sup>, Bayer CropScience) and pyroxsulam (Senior<sup>®</sup>, 750 g ai kg<sup>-1</sup>, Corteva Agriscience) (Table 1). Furthermore, the S population (as was indicated by a preliminary test in which the recommended rates of the herbicides were used) was treated with four rates [1× (recommended), 1/2×, 1/4×, 1/8×] of both ACCase- and ALS-inhibitors (Table 1). Herbicides were applied when spring milletgrass seedlings reached the 4 to 5 leaf stage and had 1 to 2 tillers (BBCH code 21-22) (Meier, 2001). Untreated plants of each population served as controls. Herbicides were applied using a portable 2.4 m boom sprayer, calibrated to deliver a water volume of 300 L ha<sup>-1</sup> at 280 kPa. Pots were kept outdoors under net protection and watered regularly. Uniform growth conditions were maintained by randomizing pot positions weekly. The plants were fertilized weekly with a commercial liquid fertilizer (Bayfolan<sup>®</sup> 11:8:6) (100 ml ha<sup>-1</sup>). A completely randomized design with three replications per treatment was used for each of the repeated experiment. Thirty-five days after treatment (DAT), plants were cut at soil level and their response to herbicide application was evaluated by measuring the aboveground fresh weight of survived plants. Two days before the fresh biomass measurement, plants were not watered.

Fresh weight data of the survived plants from the clodinafop-propargyl or diclofop-methyl treatments were used for regression analysis to determine the rates required for 50% inhibition of fresh weight ( $ED_{50}$ ) in the three spring milletgrass populations. In particular, data were fitted to a four-parameter log-logistic curve (Seefeldt *et al.*, 1995; Ritz, 2010) as follows:

$$y = c + \{[d - c]/1 + \exp[b(\log(x) - \log(ED_{50}))]\}$$

where y represents fresh weight as a percentage of the untreated control, x is the herbicide rate (g ha<sup>-1</sup> + 0.1) (the 0.1 was added because of the x equal to 0), d and c represent the upper and lower values of y, and b is the slope of the curve near  $ED_{50}$ , the rate required to halve fresh weight relative to d.

**Table 1.** Source of materials for the products used in the whole-plant rate-response experiments against the spring milletgrass (*Milium vernale*) populations

Herbicide	Trade name	Form <sup>a</sup>	Rate g ai ha <sup>-1</sup>	Manufacturer
Clodinafop-propargyl <sup>b</sup>	Sword	EC	6	K&N Efthymiadis SA, Thessaloniki, Greece
			12	
			24	
			<b>48<sup>c</sup></b>	
			96	
			192	
Diclofop-methyl	Keylofop	EC	112.5	Farma-Chem SA, Thessaloniki, Greece
			225	
			450	
			<b>900<sup>c</sup></b>	
			1800	
			3600	
			7200	

Clethodim <sup>b</sup>	Vetri	EC	30 <sup>c</sup> 60 120 <b>240<sup>c</sup></b> 480 960 1920	K&N Eftymiadis SA, Thessaloniki, Greece
Mesosulfuron- methyl + iodosulfuron methyl-sodium <sup>b</sup>	Atlantis	WG	1.87 + 0.37 3.75 + 0.75 7.5 + 1.5 <b>15 + 3<sup>c</sup></b> 30 + 6 60 + 12 120 + 24	Bayer CropScience, Athens, Greece
Pyroxsulam <sup>b</sup>	Senior	WG	2.33 4.67 9.35 <b>18.7<sup>c</sup></b> 37.4 74.8 149.6	Corteva Agriscience Hellas, Athens, Greece

<sup>a</sup>Abbreviations: EC, emulsifiable concentrate; WG, water-dispersible granules

<sup>b</sup>Clodinafop-propargyl and clethodim were applied with the surfactant Ovitex<sup>®</sup> 81.7 EC (paraffinic oil) (Arysta LifeScience Hellas S.A., Athens, Greece) at 0.5% v/v; mesosulfuron-methyl + iodosulfuron methyl-sodium and pyroxsulam were applied with the surfactant Biopower<sup>®</sup> SL (alkylethersulfate sodium salt) (Bayer CropScience, Athens, Greece) at 0.33% v/v

<sup>c</sup>The rates in boldface are the label recommended rates of the herbicides

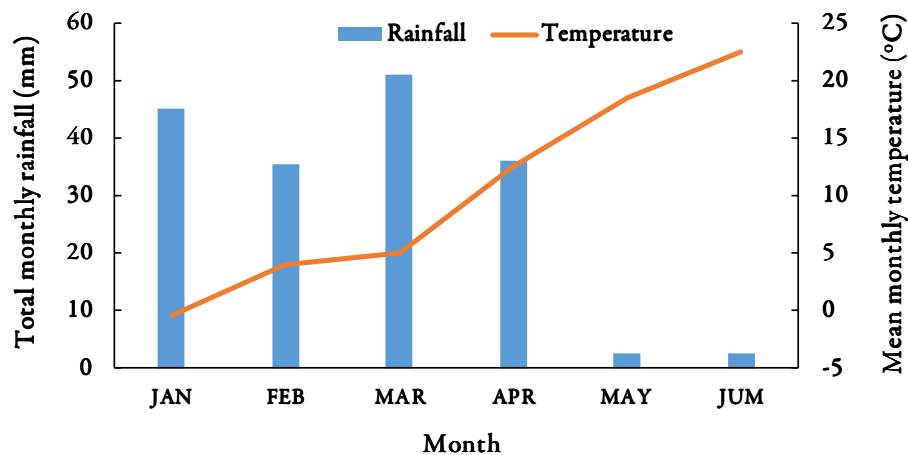
#### *Amplification and sequencing of ACCase gene fragments*

The *ACCase* gene fragment, encompassing potential mutation sites known to confer resistance to *ACCcase*-inhibiting herbicides, was amplified and sequenced in plant samples taken from the R1, R2, and S spring milletgrass populations studied in the whole-plant dose-rate assays. For gene amplification, four pots for each resistant population (R1, R2) and four pots of the S population (ten individuals per pot) were treated with the maximum labeled rate of clodinafop-propargyl, while four pots of the S population were left untreated. This procedure ensured the elimination of susceptible individuals in the R populations and confirmed the susceptibility of the S population. Leaf tissue samples from R1 and R2 plants that survived clodinafop-propargyl treatment and untreated S plants were harvested and stored at -28 °C for subsequent DNA extraction. Genomic DNA was extracted and used to amplify two DNA fragments (A and B) containing known mutation positions (1781, 1999, 2027, 2041, 2078, 2088, 2096, and 2097) linked to *ACCcase* resistance (Kaundun, 2014). The PCR reactions were performed using primers derived from blackgrass (*Alopecurus myosuroides* Huds.) (A: ACC-F1.1, ACC-R1.1; B: ACC-F2.1, ACC-R2.1) and PCR products were analyzed, purified, and sequenced using the BigDye Terminator v3.1 system. The *ACCcase* sequences from R1, R2, and S were compared with the blackgrass *ACCcase* sequence (GenBank Accession Number: AJ310767) using BioEdit v7.2.5 software (Hall, 1999).

#### *Growth rate experiment*

To examine a possible existence of fitness cost or advantage associated with the target-site mediated mechanism responsible for *ACCcase*-inhibitor resistance, a comparative growth analysis was carried out. This experiment took place from mid-winter (early January) to late spring of 2023 at the University Farm of Florina, aiming to assess the growth rates of the R1 and S spring milletgrass populations, without crop competition.

The average monthly temperature and total precipitation data recorded by the Hellenic National Meteorological Service for nearby areas are shown in Figure 1. Plastic pots (20 × 20 × 20 cm) were filled with a mixture of soil, peat, and sand, following the same procedure described in the whole-plant rate-response pot assays. Seeds of the R1 and S spring milletgrass populations were germinated in 9-cm Petri dishes with moistened filter paper and incubated in a growth chamber at 23/15 °C (day/night) under a 12-h photoperiod. Once germinated, spring milletgrass seedlings with a shoot length of around 1 cm were carefully transplanted into small jiffy pots and allowed to grow until reaching the one-leaf stage. These seedlings were then transplanted into the center of plastic pots (one seedling per pot) and maintained outdoors in a net-protected area exposed to natural environmental conditions during the experimental period. All broad-leaved or grass weeds emerging in the pots were carefully hand-removed to prevent competition with the spring milletgrass plants. Eight successive destructive samplings were conducted at 2, 4, 6, 8, 10, 12, 14, and 16 weeks after transplanting, to assess plant growth in both R1 and S populations. The final sampling coincided with the maturity of both populations. During each sampling, plants were cut at soil level and the aboveground fresh weight along with the number of tillers were recorded. The experiment followed a randomized complete block design (RCBD) with three replications (pots) for each sampling time and was repeated in time.

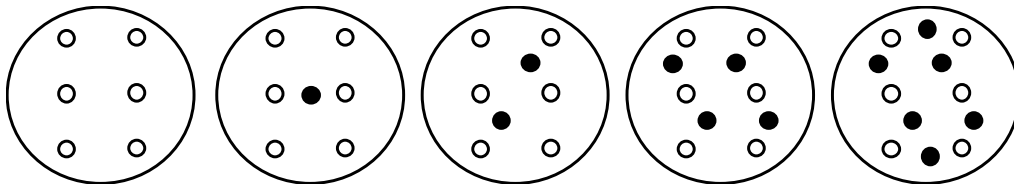


**Figure 1.** Mean monthly temperature and total monthly rainfall data recorded during January to June 2023 close to the experimental area

#### *Interspecific competition of spring milletgrass with winter wheat and barley*

A pot experiment was conducted and repeated from mid-January 2023 to the end of May 2023 at the same location and facilities, following the same procedure as the growth rate experiment. Monthly temperature and precipitation data are shown in Figure 1. A ‘target-neighborhood design’ for the crop was used to assess and compare the competitive abilities of the R1 and S spring milletgrass populations when grown in competition with winter wheat (cv. ‘Zanzibar’) or barley (cv. ‘Thessaloniki’). A template (shown in Figure 2) was used to ensure equal spacing between neighboring crop and weed plants. Initially, 12 winter wheat or barley seeds were planted in each pot in two rows, spaced 15 cm apart. Each row contained three hills, with two seeds per hill, placed 6 cm apart. When the winter wheat and barley plants reached the one-leaf stage, they were carefully thinned to leave one plant per hill, resulting in six plants per pot (equivalent to 192,000 plants ha<sup>-1</sup>). Spring milletgrass seedlings at the one-leaf stage were transplanted into the pots at five different densities: 0 (control with no weed competition), 1, 2, 4, and 6 milletgrass plants per pot (equivalent to 0, 32, 64, 128, and 192 plants m<sup>-2</sup>) (Figure 2). The pots were maintained outdoors in a net-protected area, exposed to natural conditions, and watered as needed. Fertilization was applied with 40 kg N ha<sup>-1</sup> and 50 kg P ha<sup>-1</sup> as ammonium

phosphate before sowing, with an additional 25 kg N ha<sup>-1</sup> in late February and another 25 kg N ha<sup>-1</sup> in mid-April as ammonium nitrate, following standard agronomic practices in the area to support vigorous growth of both crops and weed. Throughout the experiment, any undesired weeds emerging from the soil mixture were hand-removed. Once the crop and weed plants reached reproductive maturity, the aboveground fresh weight (a trait reflecting competitive ability), the number of tillers and the number of ears or panicles (as an indicator of potential reproductive output) were recorded to evaluate plant growth and possible reproductive outcomes. A randomized complete block design with three replications (pots) for each population and weed density treatment was used.



**Figure 2.** Schematic presentation of the density pattern (6:0, 6:1, 6:2, 6:4, 6:6) to assess growth traits of the R1 and S spring milletgrass population grown in competition with winter wheat or barley. Open circles, winter wheat/barley; black circles, R1 or S spring milletgrass populations

### *Statistical analyses*

Fresh weight data of the whole-plant rate-response experiments, expressed as a percent reduction of the untreated control, were subjected to analysis of variance (ANOVA). Especially, data derived from the rate-response experiment for the two putative resistant (R1, R2) and the susceptible (S) spring milletgrass populations were analyzed over two runs, using a 3 x 5 x 4 split-plot approach, where the three populations were considered as the main plots and the five herbicides by four rates as the subplots (Snedecor and Cochran 1989). As significant treatment by repeated (experimental) runs interaction was not found, the populations x herbicides x rates interaction means (which were significant) were averaged across two runs and compared using the Fisher's protected least significant difference (LSD) test at  $p = 0.05$ . In addition, the R software (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) was used to estimate the parameters of the log-logistic curves (Ritz and Streibig 2005), while the SAS software (Viya Platform, Statistical Analysis System, North Carolina State University) was used to test the significance of differences between parameters of the log-logistic curves (Schabenberger *et al.*, 1999).

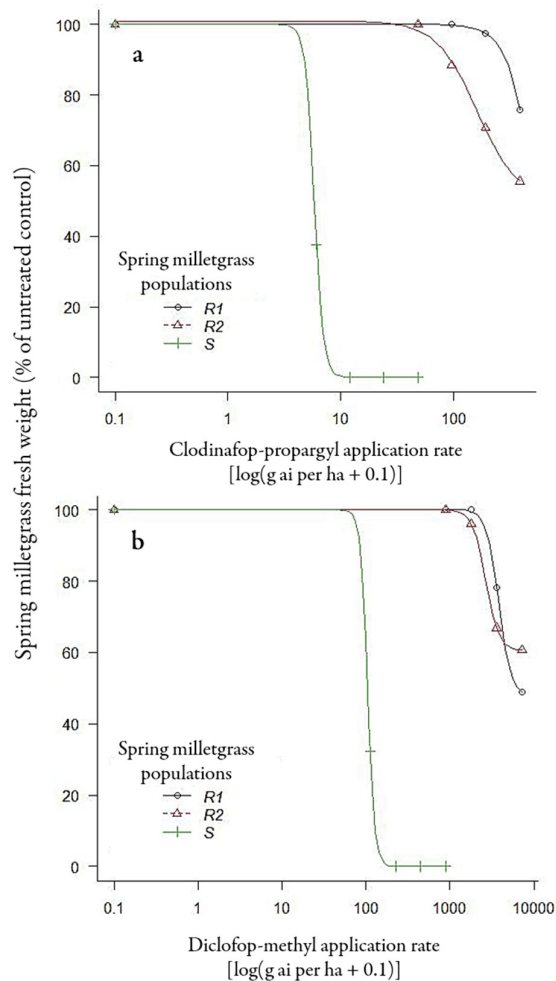
The growth rate experiment data were pooled and analyzed over the two experimental runs because the homogeneity of error variances with Bartlett's test revealed no significant departure. Therefore, a factorial approach (2 populations x 8 sampling times) was used for the combined ANOVA performed and differences between means were compared using Fisher's protected LSD test at  $p = 0.05$ .

The data obtained from the inter-specific experiments for either winter wheat or barley were also pooled together for ANOVA, following a factorial approach (2 spring milletgrass populations x 5 weed densities for either winter wheat or barley). Moreover, a factorial approach was used for spring milletgrass data (2 populations x 4 weed densities for spring milletgrass). Differences between means were compared using Fisher's protected LSD test at  $p = 0.05$ . The linear equation was also tested for its suitability to describe the relationship between fresh weight and tiller number of spring milletgrass, as well as winter wheat or barley fresh weight, tillers, and ear number response against spring milletgrass density. In these regression equations, the spring milletgrass plants per pot (density) was the independent variable (x), and the plant parameters of either weed or crop species was the dependent variable (y).

## Results

### *Whole-plant rate-response assays*

All rates (1×, 2×, 4×, 8×) of the ALS-inhibiting herbicides (mesosulfuron-methyl + iodosulfuron methyl-sodium and pyroxsulam), as well as of the ACCase inhibitor clethodim gave excellent control (100%) of the putative R1 and R2 populations (data not shown). On the contrary, the two spring milletgrass populations that survived applications of clodinafop-propargyl in small winter cereal monoculture fields were found highly cross-resistant to the aryloxyphenoxypropionate herbicides clodinafop-propargyl and diclofop-methyl. In particular, the recommended (1×) and two times the recommended (2×) field rates of clodinafop-propargyl resulted in 0% and 0 to 12% control, respectively (Figure 3a). The corresponding control provided by the 4× and 8× rates of clodinafop-propargyl ranged from 2 to 29% and 24 to 44%. Furthermore, the application of the recommended (1×) and two times (2×) the recommended label rate of diclofop-methyl resulted in 0% and 0 to 4% control, respectively (Figure 3b), while the corresponding control provided by its 4× and 8× rates ranged from 22 to 35% and 39 to 51%.



**Figure 3.** Effects of ACCase-inhibitors clodinafop-propargyl (a) and diclofop-methyl (b) application rate [ $\log(\text{g ai ha}^{-1})$ ] on fresh weight (% of untreated control) of three spring milletgrass populations (R1, R2, S). The R1 and R2 populations were exposed to 1x, 2x, 4x, and 8x rates, while the S population was exposed to 1x, 1/2x, 1/4x, and 1/8x rates. Values of each herbicide rate are means of six replicates over two runs. The parameters of the four-parameter log-logistic curves are presented in Table 2

The application of one-fourth (1/4×) of the recommended rate of all herbicides provided complete (100%) control of the S spring milletgrass population, whereas their one-eighth (1/8×) rate reduced fresh weight of this population by 62 to 100% (data not shown).

The four-parameters of the log-logistic regression analysis applied on the putative S spring milletgrass fresh weight response data to ACCase herbicides indicated that the  $ED_{50}$  values for clodinafop-propargyl and diclofop-methyl were 5.8 g ha<sup>-1</sup> and 104.8 g ha<sup>-1</sup> (Table 2), suggesting that this population was in fact susceptible to both herbicides as the  $ED_{50}$  values are lower than the 1/8× of their recommended rate. On the contrary, the putative resistant R1 population was resistant to both herbicides as the respective  $ED_{50}$  values for clodinafop-propargyl and diclofop-methyl were 681.0 g ha<sup>-1</sup> and 3837.2 g ha<sup>-1</sup>, which are higher than their 8× and 4× rates. In addition, the putative resistant R2 spring milletgrass population was moderately resistant to both herbicides as its  $ED_{50}$  values for clodinafop-propargyl and diclofop-methyl were 160.8 g ha<sup>-1</sup> and 2666.9 g ha<sup>-1</sup>, respectively, which are higher than the 2× rate. The sum of squares reduction test performed to compare pairs of regression lines showed that the parameters of the model were significantly different between the two spring milletgrass resistant populations (Table 3), suggesting that their data could not be described by a common regression line.

**Table 2.** Parameters of the four-parameter log-logistic curves describing the relationship between clodinafop-propargyl or diclofop-methyl application rate and fresh weight of three spring milletgrass populations [one susceptible (S) and two resistant (R1, R2)]

Parameters	Clodinafop-propargyl			Diclofop-methyl		
	Spring milletgrass populations					
	S (±SE)	R1 (±SE)	R2 (±SE)	S (±SE)	R1 (±SE)	R2 (±SE)
b	10.67±13.25 ns	3.41±0.25 ***	2.38±0.19 ***	10.22±17.19 ns	5.86±1.11 ***	5.53±0.31 ***
c	-0.01±0.29 ns	-94.86±150.31 ns	49.98±1.94 ***	-0.01±0.39 ns	47.79±1.18 ***	60.50±0.56 ***
d	100.00±0.46 ***	100.09±0.20 ***	100.98±0.66 ***	100.00±0.62 ***	100.18±0.38 ***	100.05±0.39 ***
<b>c (<math>ED_{50}</math>) (g ai ha<sup>-1</sup>)</b>	<b>5.81±0.34 ***</b>	<b>680.98±192.25 **</b>	<b>160.84±7.71 ***</b>	<b>104.75±12.68 ***</b>	<b>3815.27±69.89 ***</b>	<b>2666.89±54.71 ***</b>
Lower	5.11	285.81	144.98	78.68	3671.61	2554.43
Upper	6.52	1076.14	176.69	130.83	3958.93	2779.34

\*, \*\*, and \*\*\*: Significant at 0.05, 0.01, and 0.001, respectively

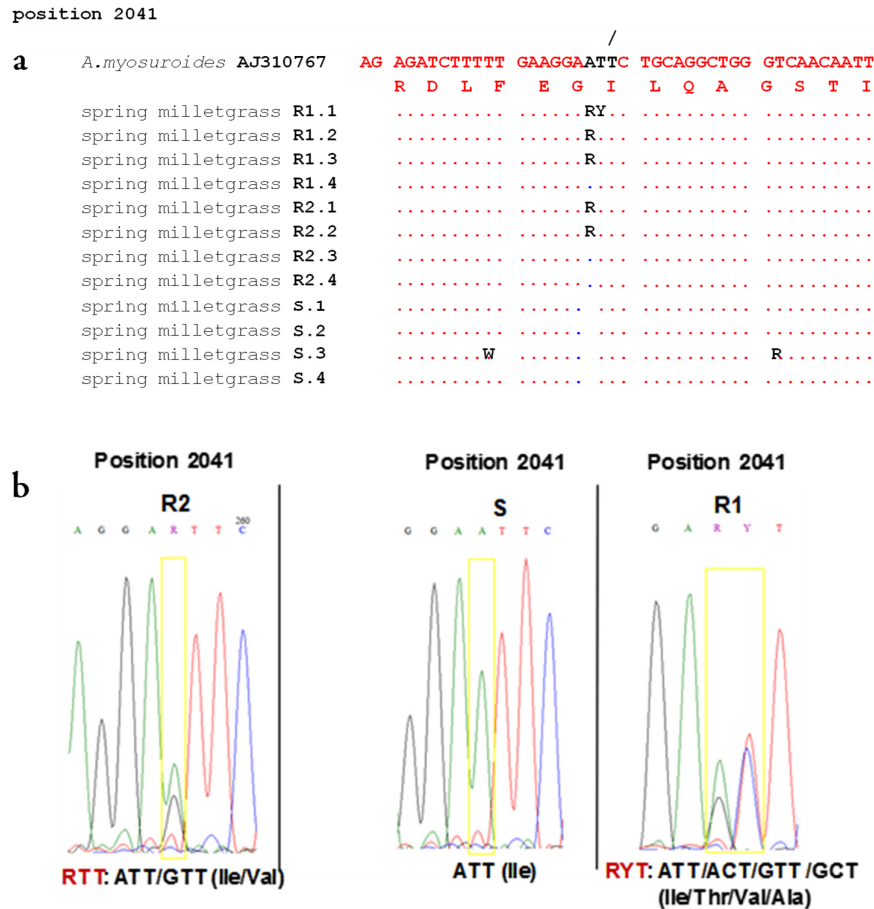
**Table 3.** Sum of squares reduction test fitting the two versions of the models to test for differences in resistance between spring milletgrass resistant populations (R1 and R2)

Biotypes	$SS_R$		$DF_R$		$MS_R$	$F_{obs}$	$p$ -value
	Full	Reduced	Full	Reduced			
<b>Clodinafop-propargyl</b>							
R1 vs. R2	383.7	3907.8	52	56	7.4	119.1	< 0.001
<b>Diclofop-methyl</b>							
R1 vs. R2	95.5	948.6	52	56	1.8	158.0	< 0.001

$SS_R$ , Sum of squares of residual;  $DF_R$ , Degrees of freedom of residual,  $MS_R$ , mean square of residual

*Amplification and sequencing of ACCase gene fragments*

Nucleotide and amino acid sequence alignment of the amplified A and B ACCase gene fragments with approximate sizes of 0.3 and 0.5 kbps showed that three and two plant samples from the R1 and R2 populations, respectively, contained heterozygous mutation(s) at position Ile2041 only (Figure 4). In particular, one plant of the R1 population contained a double heterozygous nucleotide mutation (ATT to RYT, where R:A or G and Y:T or C) converting the Ile2041 to valine (Val), alanine (Ala), and threonine (Thr), while the other two plants of this population contained an identical heterozygous point mutation (ATT to GTT) converting the Ile2041 to Val. The same heterozygous point mutation was also detected in two plants of the R2 population. Overall, homozygous nucleotide mutation was not identified in any plant samples tested at position 2041, whereas one and two resistant plants of the respective R1 and R2 populations exhibited the homozygous wild-type allele (Ile2041-Ile).

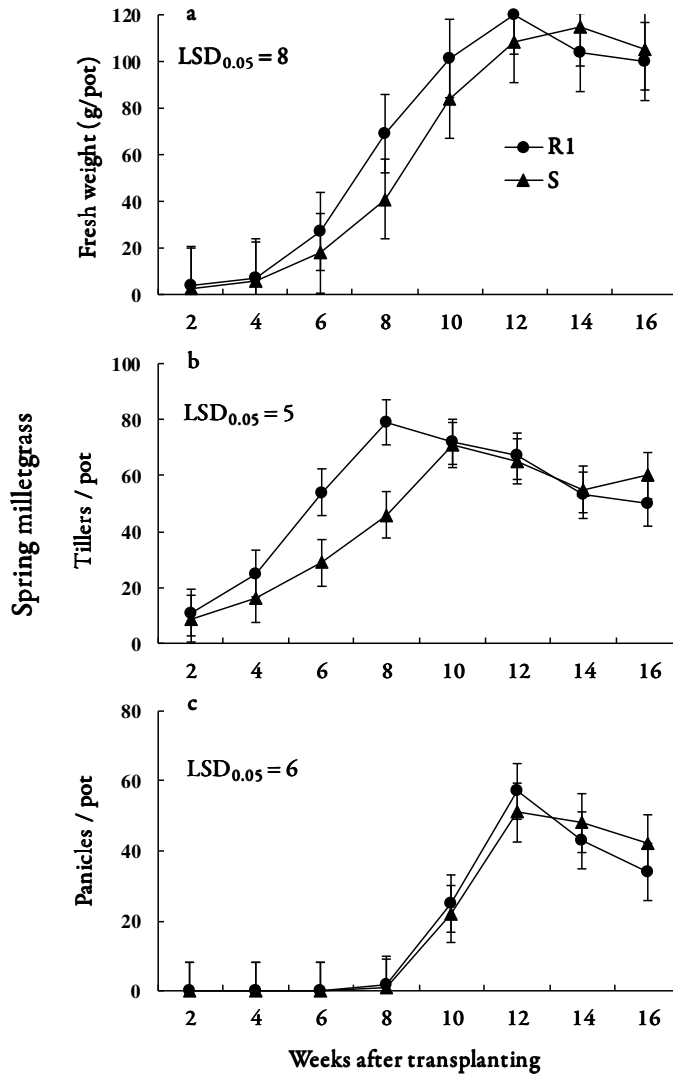


**Figure 4.** Nucleotide sequence alignment of the *ACCase* gene, as derived from plants of the S (susceptible), R1 and R2 (resistant) spring milletgrass (*Milium vernale*) populations (a) and sequence chromatograph of representative spring milletgrass plant samples at the coding region flanking codon 2041 of the *ACCase* gene (b)

The nucleotide sequences of the *Alopecurus myosuroides ACCase* gene (GenBank: AJ310767) was used as a reference. The observed point mutations at codon 2041 are highlighted in bold and correspond to nucleotide codes: ATT (Ile); R: A or G (A/G); RTT, ATT/GTT (Ile/Val); Y: T or C (T/C); RYT, ATT/ACT/GTT/GCT (Ile/Thr/Val/Ala)

#### *Growth rate experiment*

The fresh weight (Figure 5a) and tiller number (Figure 5b) of the R1 and S spring milletgrass populations grown in the absence of crop competition were significantly affected by population, sampling time and by their population by sampling time interaction. In particular, the fresh weight of the R1 and S populations followed similar trend (Figure 5a), but the R1 population produced more fresh weight than the S population between 6 and 12 weeks after transplanting. However, the tiller number of the R1 population was greater than that of the S population between 4 and 8 weeks after transplanting, but both became similar between 10 and 14 weeks after transplanting (Figure 5b). On the contrary, the panicle numbers of the R1 and S populations were similar in all sampling times (Figure 5c).



**Figure 5.** Fresh weight (a), tiller (b) and panicle number (c) produced by the ACCase resistant (R1) and the susceptible (S) spring milletgrass populations (1 plant per pot) grown in the absence of crop competition monitored by eight successive destructive samplings. Values are means of six replicates. Bars indicated the standard errors

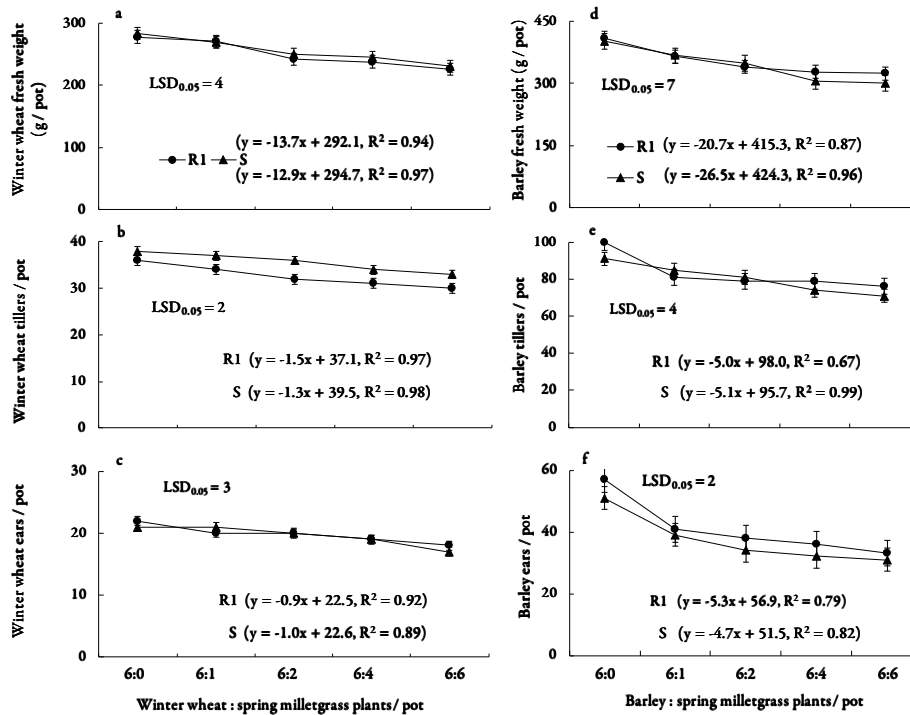
*Interspecific competition of spring milletgrass with winter wheat or barley*

The target-neighborhood design used to assess the competitive ability of the S and R1 spring milletgrass populations on winter wheat and barley indicated that fresh weight (Figures 6a, 6d), tiller number (Figures 6b, 6e) and ear number (Figures 6c, 6f) of either crop considerably decreased with increasing density of both R1 and S spring milletgrass populations. More specifically, fresh weight of winter wheat plants grown in competition with 1, 2, 4, and 6 plants of the R1 or S spring milletgrass populations was reduced by 2%, 12%, 14%, and 18% or by 5%, 11%, 13%, and 18%, respectively, compared to the weed-free winter wheat (Figure 6a). Moreover, the corresponding reduction of winter wheat tiller number due to competition of 1, 2, 4, and 6 plants of the R1 or the S spring milletgrass populations was 5%, 11%, 13%, and 16% or 2%, 5%, 10%, and 13%, respectively (Figure 6b). Furthermore, the ear number of winter wheat plants grown in competition with 1, 2,

4, and 6 spring milletgrass plants of the R1 or the S population was reduced by 9%, 9%, 13%, and 18% or by 0%, 4%, 9%, and 19%, respectively (Figure 6c).

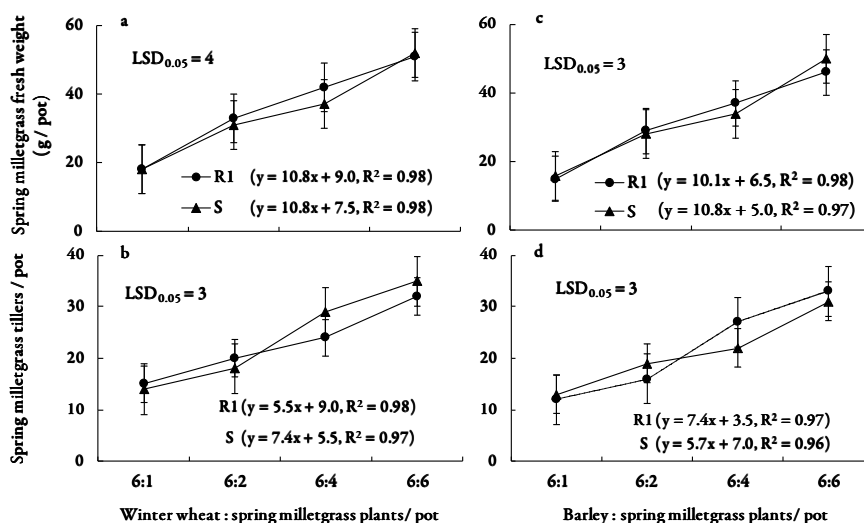
The fresh weight of barley plants grown in competition with 1, 2, 4, and 6 spring milletgrass plants of the R1 or the S populations was reduced by 10%, 16%, 19%, and 20% or by 8%, 13%, 23%, and 25%, respectively (Figure 6d), while the corresponding reduction in their tiller number was 19%, 21%, 21%, and 24% or 6%, 10%, 18%, and 21% (Figure 6e). In addition, the respective ear number reduction of the barley plants grown in competition with 1, 2, 4, and 6 spring milletgrass plants of the R1 or the S population was reduced by 28%, 33%, 36%, and 42% or by 23%, 33%, 37%, and 39% (Figure 6c).

The above results indicate clearly that fresh weight, tiller number, and ear number of either winter wheat or barley grown in competition with R1 and S spring milletgrass populations were linearly reduced with increasing weed density, and this is confirmed by the high estimated  $R^2$  values for the fitted linear equations (Figure 6). The calculated slopes for the fresh weight reduction of winter wheat or barley due to the R1 and S populations were -13.7 and -12.9 or -20.7 and -26.5, respectively (Figures 6a, 6d), while the respective ones for tiller and ear number were -1.5, -1.3, -5.0, and -5.1, or -0.9, -1.0, -5.3, and -4.7.



**Figure 6.** Fresh weight (a, d), tiller (b, e) and ear number (c, f) of winter wheat or barley (6 plants per pot) as affected by competition of the resistant (R1) and susceptible (S) spring milletgrass populations (0, 1, 2, 4, or 6 plants per pot)  
Values are means of six replicates. Bars indicated the standard errors

The fresh weight and tiller number produced by both R1 and S spring milletgrass populations grown in competition with winter wheat or barley increased linearly with increasing weed density (Figures 7a, 7c, 7b, 7d), and this is confirmed by the high estimated  $R^2$  values for the fitted linear equations (Figure 7). The calculated slopes for the fresh weight increase of the R1 and S populations due to the winter wheat or barley were 10.8 and 10.8 or 10.1 and 10.8, respectively (Figures 7a, 7c), while the respective ones for tiller number were 5.5, 7.4, 7.4, and 5.7.



**Figure 7.** Fresh weight (a, c) and number of tillers (b, d) of the resistant (R1) and susceptible (S) spring milletgrass populations (1, 2, 4, or 6 plants per pot) as affected by competition with winter wheat or barley (6 plants per pot)

Values are means of six replicates. Bars indicated the standard errors

## Discussion

### *Whole-plant rate-response assays*

The lack of control of the R1 and R2 spring milletgrass populations with higher than the maximum recommended field rates is attributed to field-evolved cross-resistance to the ACCase-inhibiting herbicides clodinafop-propargyl and diclofop-methyl, applied recurrently to manage grass weed infestations in winter cereals monoculture fields (Papapanagiotou *et al.*, 2012). This study also provides new evidence of herbicide resistance evolution in an agriculturally and economically important grass weed species infesting mainly wheat monoculture fields in northern Greece (Damanakis, 1983), as well as in other Balkan countries (Pacanoski and Mehmeti, 2019). Moreover, this is the first documented case globally of field evolved herbicide cross-resistance to ACCase-inhibitors in spring milletgrass populations grown in winter cereals. Although spring milletgrass populations evolved cross-resistance to members of the aryloxyphenoxypropionate chemical class (APPs), these populations remain sensitive to the ACCase-cyclohexanedione clethodim and to the ALS-inhibiting herbicides that provide an excellent alternative chemical control option for their management. According to other researchers (Pacanoski and Mehmeti, 2019), spring milletgrass can be effectively controlled with the preemergence applied herbicides chlortoluron plus diflufenican or pendimethalin in cereal crops. Moreover, the early detection of resistant weed plants grown in patches within fields is very critical as it allows implementation of effective management strategies over the whole farm to minimize their impact, establishment, and spread (Délye *et al.*, 2013).

The confirmed establishment of spring milletgrass populations with cross-resistance to ACCase-inhibitors, combined with the reported cross or multiple resistance of sterile oat, rigid ryegrass (*Lolium rigidum* Gaudin), and littleseed canarygrass (*Phalaris minor* Retz.) to ACCase- and ALS-inhibitors by Anthimidou *et al.* (2020), Kaloumenos *et al.* (2012), Papapanagiotou *et al.* (2012), and Travlos (2012), is anticipated as one of the greatest threats to sustainability of cereal-based production systems in Greece.

*Amplification and sequencing of ACCase gene fragments*

The Ile residue located at position 2041 is conserved in all cytosolic and chloroplastic multidomain ACCase protein sequence and plays an important role in resistance evolution to the aryloxyphenoxypropionate but not the cyclohexanedione herbicides (Délye *et al.*, 2003). Regarding the Ile2041-Val substitution, it has been reported as predominant in rigid ryegrass (Délye *et al.*, 2003) and other *Lolium* spp. populations (Scarabel *et al.*, 2011, 2020), while the Ile2041-Asn substitution has been associated with ACCase resistance in blackgrass, hood canarygrass (*Phalaris paradoxa* L.), sterile oat, and common wild oat (*Avena fatua* L.) (Beckie and Tardif, 2012). Recently, the Ile2041-Thr substitution was found in shortawn foxtail (*Alopecurus aequalis* Sobol.) to confer high levels of cross-resistance to clodinafop-propargyl and fenoxaprop (aryloxyphenoxypropionates), low resistance to pinoxaden (phenylpyrazoline) and no resistance to clethodim and sethoxydim (cyclohexanediones) (Guo *et al.*, 2017). The Ile2041-Asn mutant allele has been identified to confer high levels of resistance to aryloxyphenoxypropionates and reduced sensitivity or moderate levels of resistance to the phenylpyrazoline pinoxaden, although APP resistant grass weed populations remain susceptible to cyclohexanedione herbicides (Murphy and Tranel, 2019). Interestingly, the ACCase mutant isoform Ile2041-Val detected in rigid ryegrass did not endow high levels of resistance to the aryloxyphenoxypropionate herbicide clodinafop propargyl (Délye *et al.*, 2003), whereas in the present study it conferred high levels of resistance to spring milletgrass plants. However, Yu *et al.* (2007) found that the ACCase amino acid substitutions Ile-1781-Leu, Trp-2027-Cys, Ile-2041-Asp, Asp-2078-Gly, and Cys-2088-Arg in rigid ryegrass plants conferred cross-resistance to clodinafop, diclofop, fluazifop, haloxyfop, tralkoxydim, and pinoxaden. In addition, Saini *et al.* (2015) reported that the Ile1781-Leu and Ile2041-Asn mutations were present in seven clethodim-resistant rigid ryegrass populations, whereas the Cys2088-Arg and Gly2096-Ala mutations were identified in four and one rigid ryegrass populations, respectively.

Regarding enzyme activity and plant fitness due to ACCase mutations, Délye *et al.* (2003) and Yu *et al.* (2007) found a moderate reduction in ACCase activity due to the Ile2041-Asn substitution compared to the considerable reduction activity due to the Asp2078-Gly mutation (Darmency *et al.*, 2014).

*Growth rate experiment*

The greater fresh weight and tiller number of the R1 than the S spring milletgrass population, suggests possible fitness advantage associated with the field-selected ACCase mutation, which is in contrast with previously reported lack of association between the growth rate and the Ile1781-Leu substitution detected in the ACCase resistant blackgrass, rigid ryegrass and sterile oat (Kaundun, 2014). However, Menchari *et al.* (2008) reported that no significant adverse pleiotropic effects were found in vegetative biomass, height and seed production between wild-type and ACCase-inhibitor resistant blackgrass plants harboring the Ile1781-Leu or the Ile2041-Leu mutant alleles. On the contrary, fitness cost was determined in ACCase-inhibitor resistant blackgrass plants carrying the Asp2078-Gly amino acid substitution (Menchari *et al.*, 2008). In addition, Papapanagiotou *et al.* (2023) found that the S and the multiple R late watergrass [*Echinochloa phyllopogon* (Stapf) Koss.] populations to ALS- and ACCase-inhibiting herbicides showed similar growth traits (tiller number and fresh weight rate). Furthermore, Vila-Aiub *et al.* (2005) reported that a resistant rigid ryegrass population due to CytP450 metabolism produced less total biomass than plants of the S population or plants with target-site mediated resistance due to the ACCase Ile-1781-Leu mutation.

*Interspecific competition of spring milletgrass with winter wheat or barley*

The results of fresh weight, tiller number, and ear number of either winter wheat or barley grown in competition with R1 and S spring milletgrass populations strongly suggest a lack of association between the competitive ability of the ACCase resistant population and the underlying target-site resistance mechanism to aryloxyphenoxypropionate herbicides. This is in line with Kaundun (2014) who reported lack of association

between the shoot biomass and seed yield in ACCase-resistant blackgrass, rigid ryegrass, and sterile oat populations, and the Leu1781 mutant allele.

The similar fresh weight and tiller number of the R1 and S spring milletgrass populations grown in competition with either winter wheat or barley were expected as a consequence of the similar results obtained for the fresh biomass and tiller number of either crop.

## **Conclusions**

The results of this study indicate that field-selected spring milletgrass populations evolved high level target-site mediated ACCase cross-resistance to aryloxyphenoxypropionate herbicides, but not to ACCase-inhibiting cyclohexanediones (clethodim) or ALS-inhibiting herbicides. The resistant R1 population produced greater fresh weight and tiller number than the S population grown in the absence of winter wheat or barley competition, but both R1 and S spring milletgrass populations grown in competition with winter wheat or barley indicated similar competitive ability, suggesting lack of association between fitness and target-site mediated resistance mechanism. This study also showed that the ALS-inhibiting herbicides mesosulfuron-methyl+ iodosulfuron-methyl-sodium and pyroxsulam remain very effective alternative tools to manage the evolution of ACCase-inhibitor resistance in spring milletgrass populations infesting winter wheat monoculture fields. Also, the cyclohexanedione clethodim could be used as a highly effective chemical control option against APP resistant spring milletgrass populations in broadleaved rotational crops.

## **Authors' Contributions**

Conception and design; APP, IL and IGE. Material preparation, data collection and analysis; APP, AA, TS, IL, GM, and IV. First draft; APP, IL, and IV. Validation, reviewing, and final editing; IV and IGE. All authors read and approved the final manuscript.

## **Ethical approval** (for researches involving animals or humans)

Not applicable.

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## **Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

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