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Research Article

Johnsongrass (*Sorghum halepense* (L.) Moench) resistance to cycloxydim, fluazifop and propaquizafop and its impact on growth rate

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Abstract

One putative-resistant (R) johnsongrass (*Sorghum halepense* L. Moench) population, originating from a cotton monoculture field in northern Greece, was evaluated for the possible evolution of cross-resistance to acetyl-CoA carboxylase (ACCase)- and multiple resistance to acetolactate synthase (ALS)-inhibiting herbicides, and to elucidate the levels and underlying mechanisms of resistance. Whole-plant rate-response assays showed that the R population was highly cross-resistant to the post-emergence applied ACCase-inhibiting herbicides fluazifop-P-butyl, propaquizafop (aryloxyphenoxypropionates) and cycloxydim (cyclohexanedione), but susceptible to the ACCase-inhibitor clethodim (cyclohexanedione) and the ALS-inhibitor nicosulfuron. The analysis of the *ACCase* gene sequence revealed a point mutation (ATA to WTA/TTA) at 1781 residue in the CT domain of ACCase, resulting in an amino acid substitution from isoleucine (Ile) to leucine (Leu). However, all sequenced plants of the S johnsongrass population were found with the wild-type allele encoding Ile-1781. The R johnsongrass population, grown without competition, produced more fresh weight, rhizome biomass and number of panicles than the S population. These findings indicate clearly that the R johnsongrass population has evolved target-site cross-resistance to three ACCase-inhibitors that increased most of its growth traits as compared with the S population, suggesting a fitness advantage associated with the ACCase Leu-1781 mutation.

Keywords: *ACCase* gene sequence; ACCase inhibitors; cycloxydim; target-site resistance; weed growth rate

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Introduction

Johnsongrass [*Sorghum halepense* (L.) Moench] is an erect tetraploid, invasive perennial, predominately self-pollinated plant species that emerged from a natural crossing of *Sorghum bicolor* (L.) Moench and *Sorghum propinquum* (Kunth) Hitchc. (Paterson *et al*., 2020). It reproduces both asexually [due to below-ground storage organs (rhizomes)] and sexually. It is one of the most noxious C_4 weeds in the Mediterranean and worldwide, occurring in all major agricultural regions globally between latitudes 55°N to 45°S (Kelly *et al.*, 2020). Johnsongrass also expands its habitat continuously and causes significant losses in diverse cropping systems and in natural biodiversity globally (Reichmann *et al*., 2016; Klein and Smith, 2021).

Johnsongrass has reproduction features [production of extensively creeping rhizomes (40-90 m per plant) and large numbers of seeds (a single plant can produce as many as 80,000 seeds in one growing season)] that result in significant seedbank enrichment, increased persistence and ability to overcome implemented agronomic management strategies (Warwick and Black, 1983; Maity *et al*., 2022; González-Torralva and Norsworthy, 2024). Except for its diverse modes of propagation, johnsongrass is characterized by crop mimicry, evolving genetic diversity, tremendous vigour and plasticity, considerable adaptive capacity when faced with extreme and diverse soil and climatic conditions (exhibiting cold and drought tolerance, ability to flourish in low-fertility soils) and resistance to pathogens (Warwick and Black, 1983; McWhorter, 1989; Klein and Smith, 2021). Moreover, johnsongrass strongly competes for light, moisture and nutrients resulting in 42 to 90% yield losses in soybean (Sims and Oliver, 1990), corn (Mitskas *et al*., 2003) and cotton (Bridges and Chandler, 1987; Keeley and Thullen, 1989; Vasilakoglou *et al*., 2005). According to Uludag *et al*. (2007), even the presence of a single johnsongrass plant per m^2 can result in a considerable (7%) cotton lint yield reduction. Arable crop yields can also be negatively affected by the strong allelopathic potential of johnsongrass and its ability to serve as a host for diseases and insects (McWhorter, 1989; Vasilakoglou *et al*., 2005). Rhizomatous and seedling johnsongrass are capable of producing rhizomes soon after emergence (Mitskas *et al*., 2003).

Various chemical, cultural (such as crop rotation) and mechanical (such as summer ploughing) methods have been used for johnsongrass' management. Initially, the soil-applied thiocarbamate, dinitroaniline and chloroacetamide herbicides (effective in controlling johnsongrass seedlings, but not its rhizomes) were used. These were followed by post-emergence applied herbicides, including ACCase inhibitors (APPs and CHDs) and ALS inhibitors (mainly sulfonylureas), which offered improved control (McWhorter, 1989; González-Torralva and Norsworthy, 2024). Moreover, johnsongrass control, fecundity, seed viability and progeny are influenced by the weed growth stage at the application time of the post-emergence herbicides (Johnson and Norsworthy, 2014). Johnsongrass has a remarkable propensity to evolve resistance to most herbicide groups-modes of action employed for its chemical control (Heap, 2025; Schantz, 2025). Resistant johnsongrass populations have been detected in fields where pre-emergence or post-emergence applied herbicides were applied once or more annually over several years (Johnson *et al*., 2014; Hernández *et al*., 2015; González-Torralva and Norsworthy, 2024). In particular, johnsongrass resistance has evolved to microtubule assembly-, ACCase-, ALS- and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibitors (Smeda *et al*., 1997; Bradley *et al*., 2001; Burke *et al*., 2006a; 2006b; Kaloumenos and Eleftherohorinos, 2009; Papapanagiotou *et al*., 2022). Moreover, hybridization through pollen mediated gene flow between adjacent ALS-herbicide resistant and susceptible johnsongrass populations may spread the herbicide resistant trait to susceptible johnsongrass, which necessitates sound monitoring and management (Maity *et al*., 2022). Concerning the resistance mechanism to ACCase-inhibitors, target-site resistance [(TSR), due to single amino acid changes in the carboxyl transferase (CT) domain of the multidomain plastid ACCase found in grasses] and non-target-site resistance (NTSR) have both been reported (Kaundun, 2014; Scarabel *et al*., 2014). Mutations in the ACCase enzyme decrease its affinity to ACCase-inhibitors, conferring significant levels of resistance. On the other hand, NTSR includes a range of diverse mechanisms such as reduced penetration, impaired translocation,

sequestration and enhanced metabolism of ACCase-inhibiting herbicides (Kaundun, 2014; Scarabel *et al*., 2014).

The evolved herbicide resistance-endowing mutations result in fitness cost or advantage, under conditions of absence of selective pressure imposed by repeated herbicide applications, which is interpreted as an evolutionary perspective (Panozzo and Sattin, 2021). The possible pleiotropic effects in weed growth, reproduction and competition traits incurred by herbicide resistance depend on the specific weed species and the evolved point mutation, the genetic background, the possible fitness cost or advantage and the prevailing environmental conditions (Panozzo and Sattin, 2021). However, regarding possible fitness cost/or advantage, it is not universal for all weed species and each case must be evaluated separately (Vila-Aiub *et al*., 2009).

Cycloxydim and clethodim are the main selective postemergence herbicides widely used to control johnsongrass populations resistant to aryloxyphenoxypropionate herbicides in cotton fields of Greece (Papapanagiotou *et al*., 2022). However, cotton farmers in northern Greece complained recently for reduced efficacy of cycloxydim against some johnsongrass populations, suggesting uncertainty for johnsongrass control in cotton. Therefore, the main objectives of this study was i) to test this population for possible evolution of resistance to cycloxydim or possible cross-resistance (field-evolved resistance to herbicides with the same mode of action but belonging to different chemical groups) to other ACCase-inhibitors, ii) to evaluate the efficacy of nicosulfuron (an ALS-inhibitor) against this population, iii) to investigate whether the mechanism endowing resistance is related to an altered target enzyme and iv) to compare growth traits of the putative resistant johnsongrass population with a susceptible population.

Materials and Methods

Johnsongrass material

A population of johnsongrass suspected to be herbicide-resistant was gathered from a field (40.36184 N, 22.20522 E) in the Imathia county of northern Greece, an area where cotton has been cultivated continuously for over a decade. Inadequate control of johnsongrass following the post-emergence applications of the cyclohexanedione herbicide cycloxydim in early spring of 2022 raised concerns among farmers and agricultural specialists. To investigate potential resistance, rhizomes were collected from the surviving, actively growing johnsongrass plants during late May 2022 and subsequently used for rate-response, whole-plant pot assays (Kaloumenos and Eleftherohorinos, 2009). Additionally, records regarding prior herbicide applications and agronomic practices in the sampled field were obtained from the cotton farmer. To serve as a reference, rhizomes were also collected from johnsongrass plants growing in a non-cultivated area adjacent to the treated field (40.36238 N, 22.20504 E), which had never been exposed to herbicides and was classified as the susceptible population.

Whole-plant rate-response assays

A pot experiment was conducted in a University of Thessaloniki's farm during late spring and summer of 2022. The suspected herbicide-resistant johnsongrass population was evaluated by rate-response assays in a net-protected outdoor area to confirm evolution of herbicide resistance. Rhizomes, collected in late spring from the affected cotton field, were cut into 4-5 cm segments and planted in plastic pots (20 x 15 x 15 cm) filled with a mixture of clay loam soil, peat and sand in equal proportions. Three successfully germinated plants per pot were retained for further evaluation. Plants were irrigated as needed and fertilized weekly to maintain optimal growth.

The suspected resistant population was tested against cycloxydim (the potential selecting agent), the APP herbicides fluazifop-P-butyl and propaquizafop, the CHD herbicide clethodim and the ALS-inhibitor nicosulfuron. A suspected susceptible population, which had never been exposed to ACCase- or ALS-inhibiting herbicides, was also included in the assay. In particular, the suspected resistant population was

treated with 1x, 2x, 4x, 8x and 16x the recommended rate for each herbicide (Table 1). The susceptible population, in contrast, was exposed to the following rates 1/16x, 1/8x, 1/4x, 1/2x and 1x (recommended rate) (Table 1). Untreated control plants were maintained for both populations to enable evaluation of the herbicide efficacy. Herbicide applications were conducted when plants reached the three- to four-leaf stage (approximately 25 to 35 cm in height) using a portable field plot sprayer equipped with six 8002 flat fan nozzles (Teejet Spray System Co., Wheaton, Illinois, USA) calibrated to deliver 300 L ha^{-1} at 280 kPa pressure. All clethodim treatments included 0.5 % v/v of paraffinic oil. The whole-plant rate-response experiment was repeated in time. The experiment was established in a completely randomized design with three replications for each treatment (three pots for each repetition time x population x herbicide x rate treatment, 312 pots in total). Each pot contained three actively growing rhizomatous johnsongrass plants at the time of herbicide application. Pot randomization within each population was made weekly to ensure uniform growth conditions for all plants.

Table 1. Source of materials for the products used in the whole-plant rate–response assays against the R and S johnsongrass populations

Herbicide[a]	Trade name	Form[b]	Rates (g ai ha^{-1})	Manufacturer
Cycloxydim	Focus	EC	12.5 25 50 100 **200**[c] 400 800 1600 3200	BASF Hellas
Fluazifop-P-butyl	Fusilade	EC	15.6 31.2 62.5 125 **250**[c] 500 1000 2000 4000	K&N Efthymiadis S.A.
Propaquizafop	Agil	EC	12.5 25 50 100 **200**[c] 400 800 1600 3200	ADAMA Greece
Clethodim[a]	Vetri	EC	18.7 37.5 75 150 **300**[c] 600 1200 2400 4800	K&N Efthymiadis S.A.

Nicosulfuron	Samson Extra	OD	2.8 5.6 11.2 22.5 **45**[c] 90 180 360 720	K&N Efthymiadis S.A.

[a]Clethodim was applied with the paraffin oil 60% w/v (Atplus™, Croda International Plc, East Yorkshire, UK) at 5ml L^{-1}; [b]Abbreviations: EC, emulsifiable concentrate; OD, oil dispersion; [c]The rates in boldface are the label-recommended rates of the herbicides

Johnsongrass response was evaluated at 35 days after treatment (DAT). Weed stems were cut at one cm from the soil, and the fresh weight of surviving plants with actively growing green tissues was recorded. Fresh weight data relative to untreated controls were analyzed using an ANOVA.

Amplification and sequencing of the ACCase gene in johnsongrass

A segment of the *ACCase* gene covering potential mutation sites was amplified, sequenced and compared in both suspected resistant and susceptible johnsongrass populations. Plants were grown in pots and treated with cycloxydim at the field label rate to confirm resistance. Leaf samples from nine surviving suspected resistant plants (R) and from three untreated reference plants (S) were harvested, stored at -28 °C and used for DNA extraction. Twenty mg of leaf tissue from each individual plant was homogenized in liquid nitrogen. DNA was isolated using the NucleoSpin Plant II Mini kit (Macherey - Nagel, Germany) and its quality was assessed with a spectrophotometer (Q5000, Quawell, China). Fifty ng of isolated DNA were used for PCR amplification using the primers ACcp1 (5'-CAACTCTGGTGCTIGGATIGGCA 3') and ACcp1R (5'-GAACATAICTGAGCCACCTIAATATATT 3') developed by Délye and Michel (2005), that amplify a 551 bp part of the *ACCase* gene containing the Leu-1781 position.

A total volume of 20 μl was used for the PCR reactions, which were carried out in an Eppendorf Mastercycler thermal cycler. More specifically, 0,6 pmol of each primer, 50 ng isolated DNA, 10 μl FastGene Taq 2X Ready Mix (Nippon Genetics, Europe) and ultrapure water up to the final volume were included in each reaction. PCR conditions consisted of one step for 5 min at 94 °C, followed by 36 cycles of 30 seconds at 94 °C, 30 seconds at 56 °C and 40 seconds at 72 °C, followed by a final extension step of 72 °C for 5 min. The amplicons were initially checked in an agarose gel electrophoresis to exclude the possibility of any isomorphs or non-specific products based on the product size. The successfully amplified PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and were bidirectionally sequenced using the primer pair previously used for the PCR amplification in a 3730 automatic sequencer. The MEGA X (Molecular Evolutionary Genetics Analysis) (Kumar *et al*., 2018) software was used to identify ACCase point mutations, particularly Ile1781 [*Sorghum halepense ACCase* gene (GenBank: KF885934.1)] (González-Torralva and Norsworthy, 2024).

Johnsongrass fitness

Seed production of the R and S johnsongrass populations

Plants of the R and S johnsongrass populations were also grown in 2022 growing season for seed production needed in further studies. In particular, 30 plastic pots (30 x 25 x 20 cm), filled with a 1:1:1 mixture of clay loam soil with peat and sand, were planted with three 4-5 cm long rhizomes per pot for each johnsongrass population. All 30 pots of the R and three pots of the S johnsongrass population were sprayed with the recommended (x) label field rate of cycloxydim when plants reached the three to four-leaf stage. This application was performed to remove the possible susceptible plants from the R population and to ensure the susceptibility of the S population. The pots with the R plants were separated from the S ones at least 100 m

apart to avoid possible cross-pollination (gene flow) between the R and S plants. Both R and S plants were properly irrigated and fertilized to maintain vigorous growth throughout the growing season. Ripe seeds were harvested in late September as a bulk sample from all 30 pots of the R and 27 pots of the S johnsongrass populations, they were air-dried, threshed and maintained in a room at 5-7 °C temperature to be used in the growth rate experiment during the following year.

Growth rate comparison of the R and S johnsongrass populations

To assess potential fitness cost or advantage associated with the 1781Leu ACCase mutant allele conferring resistance, a comparative growth rate experiment was conducted in Thessaloniki during late spring to summer 2023. Therefore, the growth rates of the R and S johnsongrass populations in the absence of competition were evaluated in plastic pots (30 x 25 x 20 cm) filled with a 1:1:1 mixture of clay loam soil, peat and sand. Seeds of both populations were treated with sulfuric acid (3-4 min), followed by immersion in a 1.5% potassium nitrate solution (2 h) to enhance germination. Then, uniform seedlings were transferred to Petri dishes, moistened and incubated in a growth chamber (20/30 °C, night/day). When the johnsongrass seedlings reached approximately the one-leaf stage, they were transplanted in small jiffy pots. At the two-leaf stage, seedlings were transplanted into pots and grown outdoors under ambient conditions, where the emerged weeds in pots were manually removed. The mean monthly temperature and total monthly precipitation data in nearby area are shown in Figure 1.

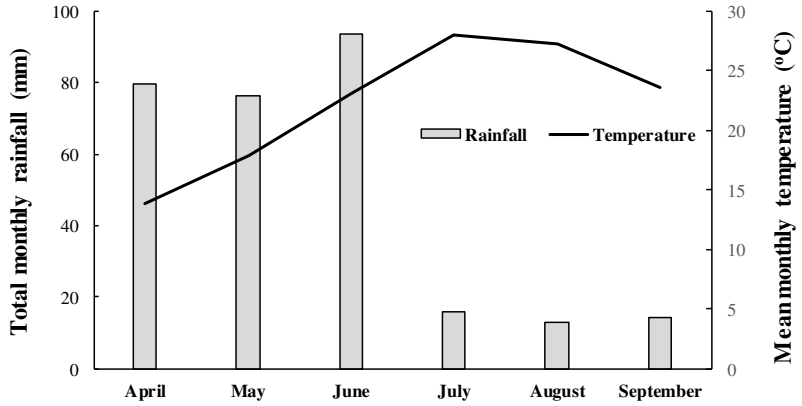


Figure 1. Mean monthly temperature and total monthly rainfall data recorded during April to September 2023 close to the experimental area

Eight destructive samplings (at 2, 4, 5, 6, 7, 8, 9 and 10 weeks after transplanting) were conducted, where tiller number, aboveground biomass, rhizome biomass and panicle numbers were determined. The experiment was also repeated in time and it was arranged in a randomized complete block design (RCBD) with three replicates (three pots per repetition time x population x sampling time, 96 pots in total).

Statistical analyses

For the whole-plant rate-response experiment data, a combined over repetition time ANOVA was performed using a split-plot approach (5 herbicides x 5 rates), separately for each population. Homogeneity of variance was verified using Bartlett's test, and mean differences were compared using Fisher's protected least significant difference (LSD) test at $p = 0.05$. Also, nonlinear regression analysis was performed using a four-parameter log-logistic equation (Seefeldt *et al*., 1995) to estimate the herbicide dose required to reduce fresh weight by 50% (GR_{50}):

$$y = c + (d - c)/\{1 + \exp[b(\log x - \log GR50)]\}$$

Where c and d represent lower and upper limits, b is the relative slope around GR_{50}, x is the herbicide dose, and y is the percentage growth response.

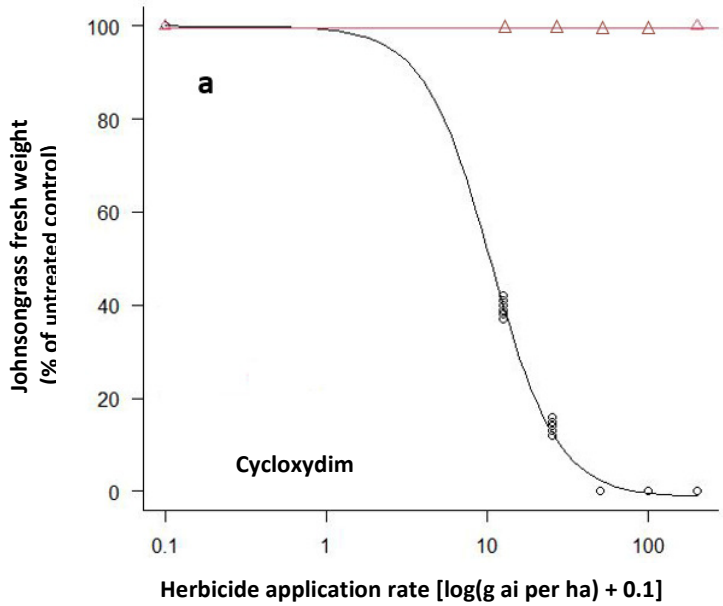
For the growth rate experiment data, an MANOVA was performed using a factorial approach (2 populations × 9 sampling times) and differences between means were assessed using Fisher's LSD test at p = 0.05. Linear regression analysis was also applied to evaluate growth trends over time.

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), the SPSS software (SPSS, 2007) was used to conduct the MANOVA, while the MSTAT-C program (MSTAT-C, 1988) was used to conduct the ANOVA.

Results

Whole-plant rate-response assays

The ANOVAs performed for the johnsongrass fresh weight data indicated no significant repetition time by treatments interactions, but significant ($p < 0.001$) herbicide by rate interactions. Thus, the herbicide by rate interactions are presented and discussed. More specifically, the application of the herbicides cycloxydim, clethodim, fluazifop-P-butyl, propaquizafop and nicosulfuron at rates of 1/4x, 1/2x and 1x (recommended rate) resulted in 100% control of the S johnsongrass population. Furthermore, the application at rates of 1/16x and 1/8x resulted in fresh weight reduction that ranged from 52% to 86% (data not shown). In contrast, the application of cycloxydim at rates of 1x, 2x, 4x, 8x and 16x reduced the fresh weight of the putative R johnsongrass population by 0%, 0%, 3%, 6% and 36%, respectively (Figure 2a). Also, the APP fluazifop-P-butyl applied at rates of 1x, 2x, 4x, 8x and 16x reduced the fresh weight of the putative R population by 11%, 22%, 29%, 36% and 55%, respectively (Figure 2b), while the respective reduction due to propaquizafop rates was 19%, 44%, 74%, 79% and 92% (Figure 2c). However, all rates of the CHD clethodim and the ALS-inhibitor nicosulfuron provided excellent control (100%) of the R johnsongrass population (data not shown).



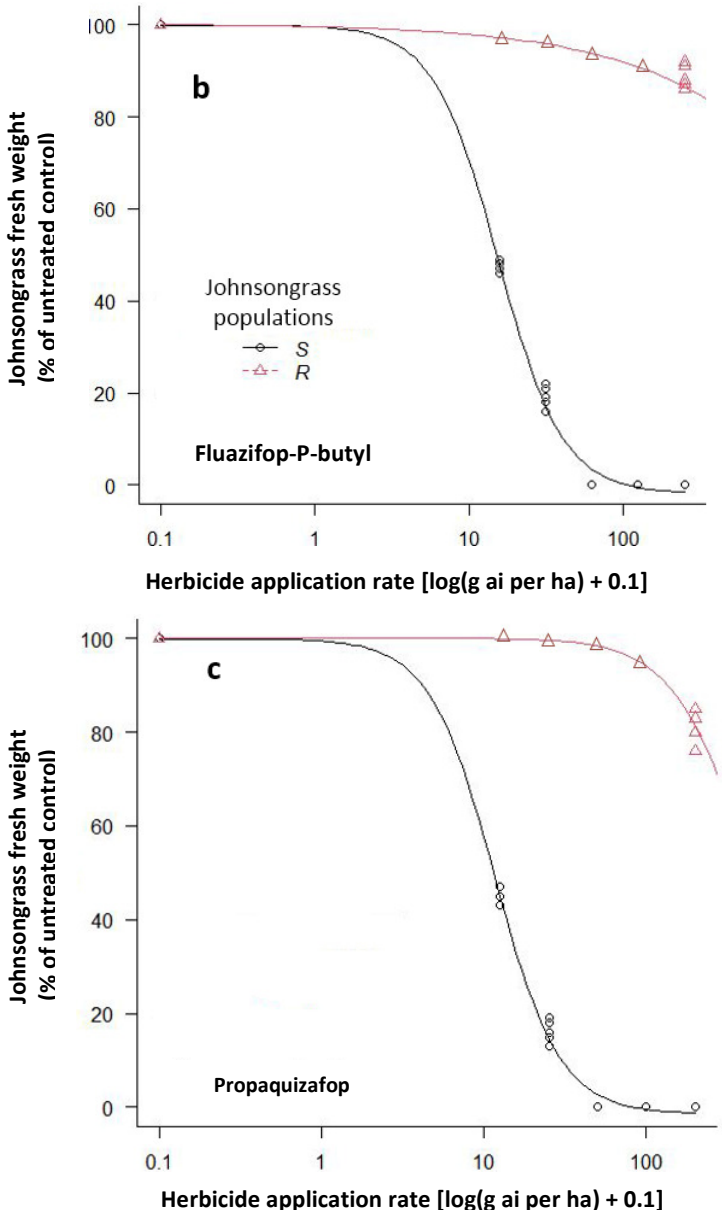


Figure 2. Fresh weight (% of untreated control) of two (S and R) johnsongrass population as affected by the ACCase-inhibiting post-emergence applied herbicides cycloxydim (a), fluazifop-P-butyl (b) and propaquizafop (c). The S population was exposed to 1/16x, 1/8x, 1/4x, 1/2x and 1x rates, while the R population was exposed to 1x, 2x, 4x, 8x and 16x rates. The parameters of the four-parameter log-logistic curves are presented in Table 2

Values of each herbicide rate are means of six replicates

Based on the estimated GR_{50} values [herbicide rate (g ai ha^{-1}) required for 50% reduction of fresh weight] for the two johnsongrass (S and R) populations (Table 2), the calculated R/S ratio for the herbicides cycloxydim, fluazifop-P-butyl and propaquizafop was 782, 2383 and 35, respectively.

Table 2. Parameters of the four-parameter log-logistic curves describing the relationship between cycloxydim, fluazifop-butyl or propaquizafop application rate and fresh weight of two johnsongrass populations [one susceptible (S) and one resistant (R)]

Parameters	Johnsongrass populations					
	Cycloxydim		Fluazifop-butyl		Propaquizafop	
	S (±SE)	**R** (±SE)	**S** (±SE)	**R** (±SE)	**S** (±SE)	**R** (±SE)
b	2.10 ± 0.09 ***	2.67 ± 0.22 ***	2.08 ± 0.09 ***	0.57 ± 0.06 ***	2.17 ± 0.09 ***	1.89 ± 0.13 ***
c	-1.25 ± 0.48 *	-380.26 ± 588.91 ns	-1.79 ± 0.66 *	-147.40 ± 129.52 ns	-1.39 ± 0.54 *	9.37 ± 1.61 ***
d	99.98 ± 0.65 ***	99.58 ± 0.36 ***	99.92 ± 0.86 ***	100.24 ± 1.14 ***	99.96 ± 0.73 ***	100.09 ± 0.38 ***
e (GR_{50}) (g ai ha^{-1})	**10.50 ± 0.18 *** **	**8214.38 ± 4418.68 ns**	**15.46 ± 0.29 *** **	**36930.0 ± 50038.0 ns**	**11.66 ± 0.19 *** **	**406.67 ± 15.55 *** **
Lower	10.14	-786.17	14.87	-64995.0	11.28	375.00
Upper	10.87	17214.93	16.05	138855.0	12.04	438.35

*, **, and ***: Significant at 0.05, 0.01, and 0.001, respectively' b - relative slope, c - lower limit, d - upper limit

Amplification and sequencing of the ACCase gene in johnsongrass

Amplification and sequencing of the *ACCase* gene fragment revealed that the nine herbicide R johnsongrass plants possessed an insensitive acetyl coenzyme-A carboxylase (ACCase) target enzyme. In particular, the *ACCase* gene fragment sequences in the nine R johnsongrass plants revealed a point mutation at the first base of the codon Ile-1781 (WTA/TTA) in the CT domain of the ACCase enzyme. The mutation resulted in the substitution of Ile-1781 by Leu with reference to the coding chloroplastic ACCase sequence of *Sorghum halepense* L. (GenBank: KF885934.1) (Figure 3). Seven out of nine ACCase-inhibitor R plants were heterozygous carrying the wild type and the mutant allele (ATA/TTA) at position Ile-1781 whereas two R plants were homozygous (TTA) (Figures 3 and 4). However, the three plants of the S johnsongrass population were homozygous for the wild type allele (ATA) at position Ile-1781.

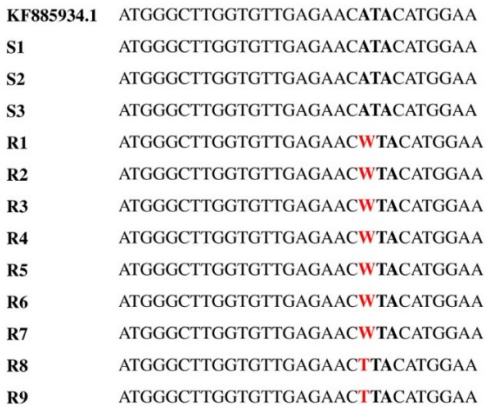


Figure 3. Alignment of johnsongrass ACCase nucleotide sequences using Molecular Evolutionary Genetics Analysis (MEGAX) software.

The codons refer to the standard sequence of the *Sorghum halepense ACCase* gene (GenBank: KF885934.1) (first sample). S1, S2, and S3 samples represent the DNA sequences of the susceptible, reference population, whereas R1 to R9 samples represent the DNA sequences of nine plants of the ACCase resistant population originating from a cotton field. The 1781 residue of the *ACCase* gene is highlighted in bold. The ATA represents the Ile amino acid, whereas the 'W' letter represents adenine (A) and thymine (T) nucleotide bases. Seven heterozygous and two homozygous R plants harbor a point mutation endowing target-site resistance due to Ile (ATA) substitution for WTA [TTA/ATA (Ile/Leu)] or TTA (Leu) at codon 1781. The R IUPAC-IUB nucleotide codes: ATA (Ile), TTA (Leu), WTA [TTA/ATA (Ile/Leu)]

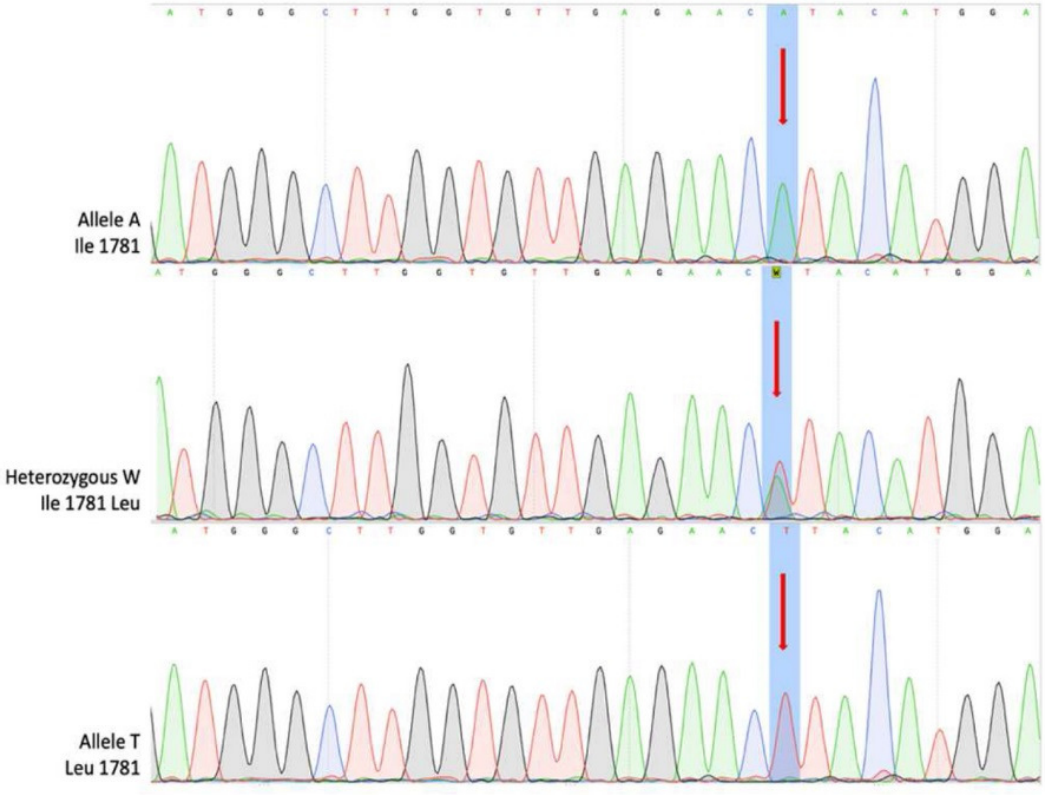


Figure 4. Point mutations were detected in the first nucleotide at 1781 codon (indicated by red arrows) at chromatograms in the analyzed *Sorghum halepense* samples. IUPAC-IUB nucleotide codes: ATA (Il), WTA [TTA/ATA (Il/Leu)], TTA (Leu)

Johnsongrass fitness

The ANOVA conducted for the johnsongrass growth-related traits [tiller number, aboveground biomass, rhizome biomass and panicle number (as an index of potential seed production)] indicated no significant repetition time by treatments interaction in most cases, but the population, sampling time and population by sampling time interactions were significant ($p < 0.001$). More specifically, most growth parameters of the R population were higher than the S population, while all parameters of both populations increased with increasing sampling time, although their increase was not proportional with sampling time (Figure 5). The linear equation provided good fit for all johnsongrass growth parameters regressed against sampling time (Figure 5), but the b slopes of the aboveground biomass, rhizomes and panicles linear equations of the R population were higher than those of the S population.

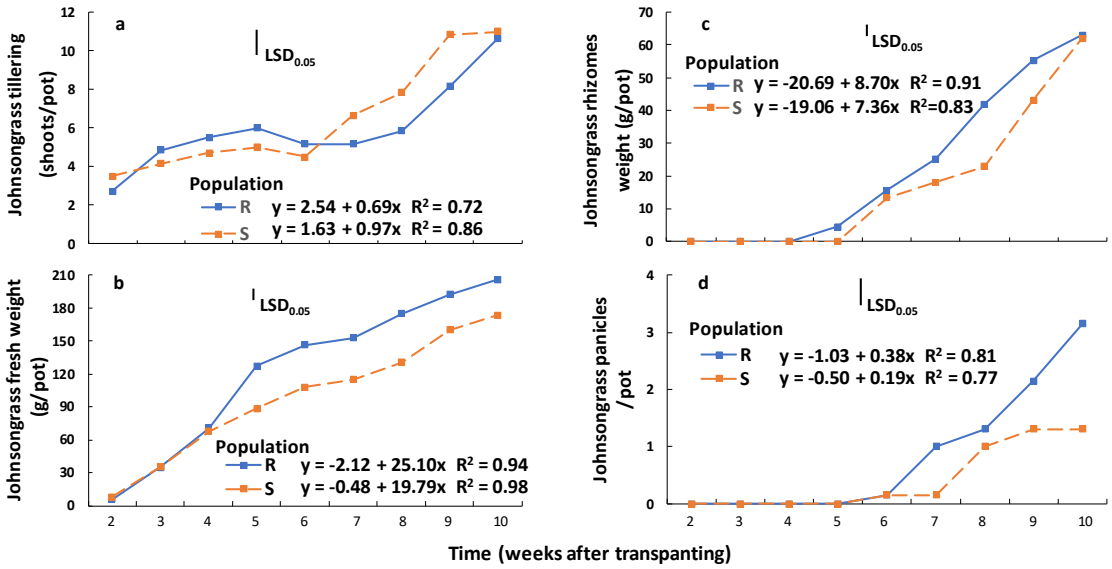


Figure 5. Tiller number (a), above-ground fresh weight (b), rhizome weight (c) and panicles (d) produced by the ACCase resistant (R) and the susceptible (S) johnsongrass populations grown in the absence of competition, monitored by eight (2, 4, 5, 6, 7, 8, 9 and 10 weeks after transplanting) successive destructive samplings

Values are means of six replicates

Discussion

Whole-plant rate-response assays

The unsatisfactory control of the field-selected R johnsongrass population by cycloxydim, fluazifop-P-ethyl or propaquizafop applied at higher than the recommended rates strongly support the evidence of cross-resistance evolution to these ACCase-inhibiting herbicides. The limited use of crop rotation and the overreliance of cotton farmers on the intense and repeated post-applied ACCase-inhibitor herbicides for effective johnsongrass control could account for the selected cross-resistance. Similar results were reported by Papapanagiotou *et al.* (2022) who found that single or repeated applications of APP herbicides in cotton grown as a monoculture resulted in the evolution of a cross-resistant johnsongrass population to ACCase-inhibiting herbicides fluazifop-p-butyl, quizalofop-pethyl and propaquizafop. In contrast, the high efficacy of clethodim against the field-selected ACCase cross-resistant population could be attributed to the different binding site of clethodim within the CT domain of the target enzyme as compared with the binding site of the APP herbicides (González-Torralva and Norsworthy, 2024).

Various herbicide resistance patterns have been identified worldwide in ACCase-herbicide resistant johnsongrass populations (Heap, 2025). Kaloumenos and Eleftherohorinos (2009) have reported that a johnsongrass population, originating from a cotton field in north-eastern Greece (county of Drama), evolved resistance to quizalofop-P-ethyl and propaquizafop, but remained sensitive to both fluazifop-P-butyl and cycloxydim. A similar resistance pattern (resistance to APP but not to CHD herbicides) has been reported in four johnsongrass populations originating from crop fields in Lombardy, Italy (Scarabel *et al.*, 2014). Similarly, Smeda *et al.* (1997) found that a johnsongrass population was resistant to fluazifop-P-butyl (388-fold), to quizalofop-P-ethyl (15-fold), moderately resistant to sethoxydim (3.4-fold) and susceptible to clethodim. Also, Bradley and Hagood (2001) reported that a johnsongrass population was 17-fold resistant to quizalofop-P ethyl and 29.5-fold resistant to fluazifop-P-butyl. The same population displayed a low level of resistance to sethoxydim but not to clethodim. Moreover, Kershner *et al.* (2012) found johnsongrass populations with high

level of cross-resistance to fluazifop-P-butyl (>64-fold) and quizalofop-P-ethyl (>54-fold) and no or negligible resistance to clethodim (1-fold) and sethoxydim (approximately 5-fold). Multiple studies across Europe and the U.S. consistently report strong resistance to APP herbicides like fluazifop-P-butyl and quizalofop-P-ethyl, with varying levels of resistance to CHDs like sethoxydim and clethodim (Bradley and Hagood, 2001; Burke *et al*., 2006a). The different cross-resistance patterns documented in various field-evolved johnsongrass populations could be attributed to diverse production systems or to different point mutations at the binding site of the ACCase or to different resistance mechanisms involved (Scarabel *et al*., 2014). Cycloxydim, until recently, was an alternative chemical option for the control of johnsongrass populations with resistance to the aryloxyphenoxypropionate chemical class of ACCase-inhibitors in Greek cotton fields. However, the emergence of field-evolved cycloxydim resistance, as confirmed in our study, along with increasing reports of its declining efficacy, signals a serious challenge for managing this weed in cotton and other broadleaf crops in Greece. This resistance dramatically limits the chemical options, leaving only clethodim among the registered selective herbicides for effective control in cotton fields. The reduced availability of water reinforced by higher prevailing temperatures and drought conditions due to climate change will undoubtedly benefit C_4 plants like johnsongrass, which has rapid adaptation mechanisms (Schantz, 2025) over C_3 plants like cotton and other broad-leaved crops such as sugarbeets, vegetables and sunflower. Moreover, climate change is expected to alter herbicide efficacy and substantially accelerate field selection of mainly NTSR-mediated resistant populations (Refatti *et al*., 2019), jeopardizing the sustainability of crop production. It is therefore crucial to achieve a better understanding of johnsongrass ecology, survival mechanisms, competitive responses and dynamics, genetic variation within and among populations, sharing weeds' adaptation and invasiveness (Smith *et al*., 2021). Moreover, it is necessary to diversify crop and weed management approaches and implement robust strategies to proactively or reactively manage johnsongrass infestations.

Amplification and sequencing of the ACCase gene in johnsongrass

The point mutation at residue 1781 (ATA to WTA/TTA) of the *ACCase* gene, found in all nine resistant plants, led to an isoleucine (Ile) to leucine (Leu) substitution. This confirmed the high level of cross-resistance observed in the rate-response assays. In contrast, the detected wild-type allele encoding Ile-1781 in all three sequenced plants of the S johnsongrass population supported the reference population's susceptibility in the rate-response assays. The Ile-1781-Leu point mutation found in this field-selected ACCase-resistant johnsongrass population was also reported by González-Torralva and Norsworthy (2024) who found that the fluazifop-resistant plants of a johnsongrass population from Arkansas-USA harbored the same mutation. According to Jang *et al*. (2013), the residue Ile1781 is a part for binding of all three classes of the ACCase-inhibiting herbicides, and for this reason the replacement of Ile with Leu or Val results in low cross-resistance factor profiles in the selected grass weed populations. However, Papapanagiotou *et al*. (2022) and Scarabel *et al*. (2014) reported that the nucleotide substitution coding for isoleucine (Ile) amino acid change to asparagine (Asn) at codon 2041 (Ile-2041-Asn) in the CT domain of *ACCase* gene in johnsongrass populations results in cross-resistance to APP herbicides, while Kershner *et al*. (2012) detected in ACCase R johnsongrass populations the Trp to Cys amino acid substitution at position 2027 (Trp-2027-Cys). Unraveling the underlying mechanism(s) of herbicide resistance is critical, allowing understanding and estimation of selection establishment, evolutionary dynamics of resistance and severity of infestations. It is also crucial for implementing effective management strategies and mitigating the spread of field-evolved herbicide-resistant populations (González-Torralva and Norsworthy, 2024).

Non-target-site resistance (NTSR) seemed to be less probable mechanism responsible for the evolved ACCase resistance, because target-site mediated resistance is usually associated with significantly higher resistance factors than those reported for metabolism-based resistance (Kaundun, 2014). It is worth noting that the overproduction of the target enzyme (increased ACCase-specific activity) as being responsible for resistance evolution in a johnsongrass population has also been reported (Bradley *et al*., 2001).

Johnsongrass fitness

The greater performance in growth and reproductive traits (fresh weight, rhizome production and number of panicles) of the R compared to S population, along with their higher slopes estimated from the linear equations fitted to these parameters against sampling time, suggest a fitness advantage of the R population harboring the field-selected ACCase allelic variant Ile-1781-Leu. As reported by Wang *et al*. (2021), populations of johnsongrass differed in their biomass when individuals were growing alone, but not when they were grown either under interspecific or intraspecific competition and assumed a trade-off between individual growth and competitive ability. The R population allocated a higher proportion of biomass in panicle production and belowground biomass, displaying an allocation strategy that will allow its increased persistence, establishment and aggressiveness in the agroecosystem. Higher aboveground biomass and panicle production clearly indicate a potential higher seed output, strongly suggesting no adaptive cost on the R population and higher fitness and competitiveness compared to its S counterpart. The increased rhizome production and weight determined in the R population could be advantageous in environments with low resource availability (Reichmann, *et al*., 2016). High reproductive capacity and lack of a fitness penalty in the absence of intra- or interspecific competition suggest that the R population will have a considerable advantage and increased evolutionary dynamics. This will be strongly expressed under both recurrent herbicide applications and in the absence of ACCase-inhibiting herbicides. Moreover, Lauenroth and Gokhale (2023), in their population-based deterministic model, have reported that in johnsongrass, target-site mutations exist with very low or even no fitness cost. These findings are in agreement with the results of Wang *et al*. (2010) who found that this resistant allele can even result in an increase in several growth components (early growth, flowering, panicle production and seed output). Moreover, Menchari *et al*. (2008) reported that the ACCase-resistance endowing Ile1781Leu substitution is not associated with adverse pleiotropic effects neither vegetative nor reproductive plant traits. The advantage of the 1781Leu mutant compared to the wild-type allele was attributed to the presence of a tightly linked ACCase, an advantageous gene with higher fitness in the R biotypes (Kaundun, 2014). Also, the delayed germination associated with the Ile1781Leu mutation determined in blackgrass (*Alopecurus myosuroides* Huds.) was interpreted as an effective means to avoid early-season herbicides and other weed management practices, leading to higher survival rates (Délye *et al*., 2013). In contrast, Panozzo and Sattin (2021) found that an APP resistant johnsongrass population due to the Ile-2041-Asn target site mutation of the *ACCase* gene displayed higher production of rhizomes but significantly lower (by 30%) production of seeds than the S population.

Johnsongrass is affected more by intraspecific competition than by interspecific competition from other weed species or crop plants (Mitskas *et al*., 2003). Therefore, the increased adaptive and competitive ability of the R plants with the target-site Ile1781Leu mutation could probably provide clear growth advantage as compared to the S population. Therefore, once the resistant-endowing mutant 1781Leu allele is field-selected, it is possible to display rapid and effective dispersion and could eventually dominate the wild type populations in the arable field crops (Vila-Aiub *et al*., 2009). Sexual reproduction including cross-pollination (pollen mediated gene flow) and flowering synchrony between different populations of johnsongrass allows the successful introgression of allele(s)-conferring traits into new genetic backgrounds with growth advantage, which may increase the ability of johnsongrass to rapidly invade, adapt, persist and thrive in new environments (Maity *et al*., 2022). It is worth mentioning that cross-pollination is affected by the size of both pollen-donor and recipient johnsongrass populations and by prevailing environmental factors such as temperature, humidity, speed and direction of wind gusts (Maity *et al*., 2022).

Based on the findings of this study, future research should be focused on determining the relative fitness of the S and R ACCase-inhibitor resistant populations grown in competitive interaction with summer crops (cotton, corn, sunflower), to highlight their vegetative and reproductive performance, reveal possible pleiotropic fitness costs/advantages and also study their intraspecific competition. Nevertheless, it is crucial to

adopt diversified, complementary, both chemical and non-chemical practices for effective and sustainable johnsongrass control and for minimizing the risk of herbicide resistance evolution. In terms of chemical control, it is necessary to avoid repeated selection pressure exerted by ACCase-inhibiting herbicides. In addition, implement herbicide rotation and, in general to reduce reliance on herbicides as the exclusive means of weed management. The application of non-selective pre-sowing herbicides could also contribute to johnsongrass chemical control. Other strategies for johnsongrass management can include systematic crop rotation (alternating between winter and summer crops), limitation or avoidance of conservation tillage, and stale seedbed preparation. In addition, summer fallowing and frequent mowing aim to weaken johnsongrass plants, reduce rhizome growth and deplete carbohydrate storage. Also, repeated plowing for consecutive growing seasons allows desiccation during summer and high winter mortality of rhizomes. Moreover, constant weed monitoring for early recognition of the initial presence of putative resistant plants. These early escapes within treated fields need to be effectively managed with chemical applications or manual weeding before they develop large patches of an aggregated pattern and high densities. These site-specific treatments can reduce the likelihood of establishment and further spread of ACCase mutant alleles in crop fields and retard or even reverse the widespread invasion of johnsongrass in cropping systems.

Conclusions

The findings of this study supported the evidence of evolved cross-resistance to the cyclohexanedione cycloxydim and to the aryloxyphenoxypropionate herbicides fluazifop-p-butyl and propaquizafop in a johnsongrass population selected from a cotton field located in northern Greece, where cotton has been cultivated continuously for over a decade with overreliance of cotton farmers on the intense and repeated post-applied ACCase-inhibitor herbicides for effective johnsongrass control. The *ACCase* gene sequencing revealed target-site cross-resistance to herbicides due to an isoleucine (Ile) to leucine (Leu) substitution at codon 1781 of the ACCase enzyme. The 1781Leu *ACCase* mutant allele conferring cross-resistance to johnsongrass population displayed higher growth rate traits compared to the S population, suggesting that the target-site mediated resistance mechanism due to the 1781Leu mutant allele is associated with a growth advantage. However, as both R and S populations remained susceptible to the ACCase-inhibitor clethodim and the ALS-inhibitor nicosulfuron, this suggests their use as alternative chemical options for their control in arable broad-leaved and corn crops, and also as effective tools, in combination with crop rotation, cultural and mechanical practices, which could reduce the selection, establishment, spread of johnsongrass infestations and mitigate ACCase-inhibitor resistance in the arable fields.

Authors' Contributions

Conceptualization: AP, IE; Methodology: AP, IG; Statistical analysis: IV; Resources: AP; Data curation: AP, MA, IG; Writing-original draft preparation: AP, IV; Writing-review and editing: IV and IE.

All authors have read and agreed to the published version of the manuscript.

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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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