**Short title: ALS-resistant redroot pigweed**

**Field-evolved cross-resistance to ALS inhibiting herbicides in redroot pigweed (*Amaranthus retroflexus* L.) populations and alternative chemical options for effective control**

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**Abstract**

Twelve putative-resistant (R) redroot pigweed populations were collected in sunflower and soybean fields located in northeastern Greece, after repeated exposure to the acetolactate synthase (ALS)-inhibiting herbicides imazamox and tribenuron-methyl. Studies were conducted to determine the resistance status to these two ALS-inhibiting herbicides and evaluate alternative post- and preemergence herbicides for effective control. Two susceptible (S) populations were also included for comparison. Eleven out of the 12 putative-R populations studied in the whole plant dose response pot experiments were characterized as cross-resistant (R) to the imidazolinone imazamox and the sulfonylurea tribenuron-methyl. In contrast, the putative R5 and the two reference populations (S1, S2) populations were found susceptible. Sequencing of the *ALS* gene revealed that a point mutation (TGG to TTG at position 574) was selected in domain B, where in combination with domain A the majority of point mutations conferring resistance have been detected, resulting in an amino acid substitution from tryptophan (Trp) to leucine (Leu) in the 11 R populations. By contrast, all sequenced plants of the three susceptible populations were found with the wild-type allele encoding Trp574. The labeled rate of the postemergence herbicides tembotrione and dicamba provided fair to excellent control of the populations with ALS cross-resistance. In contrast, at this rate the preemergence herbicides *S*-metolachlor+terbuthylazine, isoxaflutole, aclonifen, metribuzin, and pendimethalin provided excellent control. These findings strongly suggest that 11 redroot pigweed populations have evolved cross-resistance to ALS-inhibiting herbicides, but viable options for chemical control of this weed still exist.


**Nomenclature:** aclonifen; dicamba; imazamox; isoxaflutole; metribuzin; pendimethalin; *S*-metolachlor; tembotrione; terbuthylazine; tribenuron-methyl; redroot pigweed, *Amaranthus retroflexus* L. AMARE; soybean, *Glycine max* (L.) Merr.; sunflower, *Helianthus annuus* L.


**Key words:** ALS-inhibitors; weed resistance; target-site resistance

**Introduction**

Redroot pigweed is native to central and eastern USA, adjacent regions of northeastern Mexico, and southeastern Canada (Sauer 1967). In general, it is widely distributed across the temperate regions of the southern and northern hemispheres. Redroot pigweed consists of numerous ecotypes that differ significantly in morphological and biochemical traits due to high genetic heterogeneity, exhibiting various responses to herbicides, other abiotic or biotic stresses. It is a summer annual, monoecious, and self-pollinated species (Brenner et al. 2000), with occasional outcrossing (Holm et al. 1997). It is a prolific seed producer capable of producing 100.000 seeds per plant (Weaver and McWilliams 1980), largely depending on prevailing growing conditions. Redroot pigweed seeds are characterized by considerable longevity, and frequently display annual changes in dormancy status. It tolerates a wide range of soil types, textures, and pH levels (4.2 to 9.1), although it thrives in disturbed loamy, sandy-loam or silty-loamy, nutrient-enriched soils, with high levels of organic matter and nitrogen content (Dieleman et al. 2000).

Redroot pigweed is ranked among the world's worst weeds as it infests a wide range of at least 60 field and horticultural row crops in 70 countries (Holm et al. 1997). As it tolerates highly unstable environments, it can effectively compete with other plants for nutrients, water, light, and space utilizing a wide array of different beneficial survival tactics (Moghadam et al. 2021). In general, the great adverse effects of redroot pigweed populations on irrigated summer row-crop production systems (cotton, corn, soybean, sunflower) could be attributed to this species' increased ability to survive, establish, and successfully reproduce, along with a fast growth rate due to its C4 plant physiology that enables rapid adaption to diverse agroecosystems (Assad et al. 2017; Scarabel et al. 2007).

Crop interference from redroot pigweed and other *Amaranthus* species results in detrimental reductions in crop yield and quality, compromised harvest efficiency, toxicity to livestock, and release of allelopathic compounds responsible for hampering the growth and development of crop plants (Athanassova 1995). Serious losses have been documented in cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.), sorghum (*Sorghum* bicolor (L.) Moench), peanut (*Arachis hypogaea* L.), and vegetable crops (Bukun 2011; Ma et al. 2015; Moore et al. 2004). Yield losses in corn and soybean can reach approximately 90% when redroot pigweed and other *Amaranthus* species occur at high density (over 30 plants m$^{-2}$) (Costea et al. 2004). Yield losses due to redroot pigweed interference are generally greater when weed plants emerge early in the crop cycle (Mirshekari et al. 2010) and when crops like cotton have low density of established plants or display slow initial growth.

Weedy *Amaranthus* species are very prone to evolving herbicide resistant populations, and this is confirmed by the fact that field-selected herbicide-resistant redroot pigweed and the closely related species *A. hybridus* and *A. powellii* have been found in different crop fields and production systems worldwide (Heap 2024). Resistant populations within each crop field usually evolve independently in response to intense selection pressure due to repeated use of the same herbicide or herbicides with the same site of action. The constantly increasing reliance on highly effective target-site specific herbicides such as acetolactate synthase (ALS) inhibitors unavoidably results in field-selection of herbicide-resistant weed populations. Since their initial discovery and introduction, ALS inhibitors were enthusiastically adopted in most cropping systems worldwide due to a range of desirable characteristics, such as excellent efficacy at low rates, broad spectrum of activity against numerous weeds, good crop selectivity and low mammalian toxicity (Scarabel et al. 2007). ALS inhibitors were extensively and repeatedly used in most arable crops to effectively control several important grass and broad-leaved weed species. Unfortunately, this specific site of action is by far (compared to other herbicidal sites of action) the most prone to rapid selection of resistant weed populations (Heap 2024). Resistance to ALS-inhibiting herbicides frequently evolved in redroot pigweed (Chen et al. 2015; Francischini et al. 2014; Mcnaughton et al. 2009; Scarabel et al. 2007), and other *Amaranthus* species (Ferguson et al. 2001). Field-evolved ALS-inhibitor resistance is in most cases target-site mediated, which is conferred by point mutations in the *ALS* gene, resulting in an altered target enzyme that exhibits decreased sensitivity to the ALS-inhibiting herbicides. However, Cao et al. (2021) found a redroot pigweed population highly cross-resistant to thifensulfuron-methyl and other dissimilar ALS-inhibitors (imazethapyr, flumetsulam, bispyribac-sodium) due to field-selected target-site mutations probably coexisting with enhanced cytochrome P450-mediated metabolism (non-target-site resistance, NTSR). In addition, Yang et al. (2023) reported that enhanced herbicide metabolism (cytochrome P450s- and glutathione S-transferases-based) and target-site mutations confer multiple ALS- and protoporphyrinogen oxidase (PPO)-inhibitor resistance in a redroot pigweed population originated from a soybean field. Moreover, multiple resistance to triazines (atrazine, prometryn) and ALS-inhibitors (trifloxysulfuron-sodium, pyrithiobac-sodium) was reported in various redroot pigweed populations infesting cotton fields in Brazil (Francischini et al. 2019). Jones et al. (2023), Wang et al. (2017), Wang et al. (2019), and Yang et al. (2023) also found a redroot pigweed population with multiple resistance to ALS and PPO inhibiting herbicides, conferred by field-selected point mutations in the target enzymes. Finally, Du et al. (2021) and Huang et al.

(2020) reported field-evolved, target-site mediated resistance to fomesafen (PPO inhibitor) in redroot pigweed populations infesting soybean fields in China.

During the 2020-2021 growing season, some farmers in northeastern Greece reported unsatisfactory control of redroot pigweed infestations in summer row crops (soybean and sunflower) following the application of the imidazolinone herbicide imazamox and the sulfonylurea tribenuron-methyl. Both ALS-inhibiting herbicides had been repeatedly used in sunflower and soybean crops to manage weed infestations for many consecutive years. The objectives of this study were to (1) test the putative resistant (R) redroot pigweed populations for resistance evolution to the ALS-inhibitors imazamox and tribenuron-methyl, (2) elucidate the molecular mechanism conferring ALS-resistance, and (3) evaluate the efficacy of other post- and preemergence herbicides with different sites of action registered in sunflower, soybean, and corn for effective redroot pigweed control.

## Materials and Methods

### *Seed source of ALS putative resistant redroot pigweed populations*

A roadside survey was conducted in northeastern Macedonia, Greece (Kavala region), during late summer of the 2021 growing season in sunflower and soybean fields with a history of at least 10 consecutive years of applications with imazamox and tribenuron-methyl, following farmers complains for reduced redroot pigweed control by the above mentioned herbicides. Twelve redroot pigweed populations were collected from fields showing ALS-failure before crop harvest. Field selection was based on the most notable presence of redroot pigweed escapes. Mature seeds were collected by hand from 80-90 individual redroot pigweed plants taking care to obtain a representative sample from each field. The collected seeds from all plants per field were pooled together leading to 12 seed lots which were treated as populations with putative resistance (R1-R12). For comparison, two susceptible populations (S1, S2) were included in the trials as well, collected from redroot pigweed plants grown in two non-cultivated areas, away from field margins, that were never exposed to herbicide applications. The coordinates of sampling location for each population are shown in Table 1. The collected seeds were transferred to the laboratory, air-dried, threshed, placed in paper bags, and stored at room temperature (18-25$^{o}$C) to be used in the subsequent experiments.

### *Whole plant assay to postemergence herbicides*

Two identical pot trials were conducted at different times in a net-protected area at the farm of Western Macedonia, Greece in the prefecture of Florina during late spring-early summer

2022 to evaluate the 12 putative R (R1-R12) and two susceptible (S1, S2) redroot pigweed populations for possible evolution of cross-resistance to the imidazolinone imazamox and to the sulfonylurea tribenuron-methyl (ALS-inhibitors) registered for use in soybean "Clearfield" sunflower and "Express Sun" sunflower crops in Greece, respectively. In addition, all these populations were evaluated for possible multiple resistance to benzoic derivative dicamba (synthetic auxin) and triketone tembotrione (4-Hydroxyphenylpyruvate dioxygenase (4-HPPD) inhibitor), registered for use in corn fields in Greece. The experiment was conducted in 0.9L plastic pots filled with a 1:1:1 (v/v/v) mixture of clay loam soil, peat and sand. Each pot was surface seeded with about 25-30 seeds of redroot pigweed seeds and carefully covered by a thin layer (0.5-1.0 cm) of the same soil/peat/sand mixture. At the two-leaf growth stage, emerged redroot pigweed seedlings were carefully thinned to six per pot. All pots were placed outdoors, irrigated, and fertilized with a liquid fertilizer (Bayfolan®️ 11:8:6) as required for optimum plant growth. The redroot pigweed plants of all putative R and S populations were sprayed with the selected herbicides at the three- to four leaf growth stage, corresponding to growth stage 13-14 of the extended BBCH Scale (Scarabel et al., 2007), as recommended in the label of these herbicides in Greece. More specifically, populations were treated with the recommended ($1\times$), twice ($2\times$) and four times the recommended ($4\times$) maximum field rates of imazamox, tribenuron-methyl, tembotrione and dicamba. A nontreated control was also included. All herbicide rates as active ingredients, along with corresponding trade names, formulations and manufactures are presented in Table 2. A propane pressurized field plot sprayer (AZO-Sprayers, BJ Ede, Netherlands) equipped with a 2.4-m wide boom, was used for the herbicide applications. The sprayer carried six 8002 flat-fan nozzles and delivered 300 L ha$^{-1}$ of water at 280 kPa. The 12 putative R and two S populations were evaluated in the same run, and the experiment was conducted twice. The experimental design for each of the two identical whole plant pot experiments was a randomized complete block design with three replications per herbicide rate. Pot randomization within each population was made weekly to ensure uniform growth conditions for all plants. Redroot pigweed control was evaluated by determining the aboveground fresh weight of the surviving plants at 35 days after treatment (DAT). The susceptible dead plants with only dead biomass corresponded to zero fresh weight. The fresh weight data were expressed as a percent reduction of the nontreated control and subjected to analysis of variance (ANOVA).

### Whole plant assay to alternative preemergence herbicides

A whole plant pot experiment was carried out during late spring-early summer of 2023 at the farm of the University of Western Macedonia, Florina, to assess the response of 14 redroot pigweed populations (R1-R12 and S1, S2) to five residual preemergence applied herbicides with alternative sites of action. The selected herbicides included *S*-metolachlor [very long chain fatty acid (VLCFA) inhibitor] + terbuthylazine [photosystem II (PS II) inhibitor] and isoxaflutole (4-HPPD inhibitor) registered for use in corn, aclonifen [solanesyl diphosphate synthase (SPS) inhibitor] and metribuzin (PS II inhibitor) registered for use in sunflower and soybean, respectively, and pendimethalin (microtubule assembly inhibitor) registered for use in corn, cotton, sunflower, and soybean (Table 3). The experiment was established in 0.9 L plastic pots filled with a mixture of clay loam soil with sand (4:1, v/v). Each pot was seeded with approximately 25 to 30 redroot pigweed seeds and carefully covered with a layer of 0.5-1 cm of the soil/sand mixture. The 12 putative R and two S populations were treated with the recommended (1×) and 2× maximum field label rates of the previously mentioned residual soil-applied herbicides. All herbicide rates as active ingredients, along with corresponding trade names, formulations, and manufacturers, are presented in Table 3. A nontreated control for each population was also included. The application of the tested alternative herbicides was performed by the same field plot sprayer as mentioned previously. After preemergence treatments, all pots were uniformly irrigated with a water volume corresponding to 10mm of rainfall to incorporate the residual herbicides into the soil mixture. All pots were randomly placed outdoors in a net-protected area and were watered and fertilized as needed. Pots were re-randomized each week to achieve uniform growth conditions for all plants. Redroot pigweed control was evaluated by determining the aboveground fresh weight of emerged plants after preemergence applications at 40 days after treatment (DAT). The fresh weight data were expressed as a percentage of the nontreated control for analysis of variance (ANOVA).

### Amplification and sequencing of the ALS gene fragment

As the whole plant dose response assays to postemergence herbicides indicated that 11 out of the 14 evaluated redroot pigweed populations were cross-resistant to imazamox and tribenuron-methyl, further studies were conducted to confirm and characterize the nature of the molecular mechanism conferring ALS cross-resistance to the 11 field-selected weed populations. Therefore, the *ALS* gene fragment covering potential mutation sites in the 11 R (R1-R4, R6-R12) and three susceptible (S1, S2, R5) redroot pigweed populations was

amplified, sequenced, and compared. For the amplification of the *ALS* gene, plant material was collected from redroot pigweed individual plants, grown in three pots per each of the 11 R redroot pigweed populations and in six pots of the three susceptible (S1, S2, R5) populations. The 33 (11×3) pots with the resistant and the nine (3×3) pots with the susceptible plants were established and treated as previously for the whole plant assays, with the labelled field rate of imazamox, while the other nine (3×3) pots from the three susceptible populations were left nontreated. The herbicide application was made to eliminate individual S plants from the R populations and ensure the susceptibility of the S redroot pigweed populations. Leaf tissues from three surviving redroot pigweed plants in the pots of each R population and three plants from each susceptible untreated population (S1, S2, and R5) were harvested, immediately stored at -28 C, and subsequently used for DNA extraction. Twenty mg of leaf tissue from each individual plant was weighed and homogenized in liquid nitrogen. Isolation of genomic DNA was performed from each homogenate using the NucleoSpin Plant II Mini kit for DNA (Macherey – Nagel, Germany), following the manufacturer's guidelines. Isolated DNA quality and concentration were evaluated in a Q5000 spectrophotometer (Quawell, China). Fifty ng of isolated DNA were further utilized for amplification of the *ALS* gene using the primer pairs GC-ALS-197 (5′-AGGTGGAGCTTCAATGGAGA-3′) – AMA-2R (5'-CTTCTTCCATCACCCTCTGT-3') and AMA-3F (5-AACAGGTCCAGGTCTACCAGA-3) – GC-ALS574 5′-CCTGCAGGAATCATGGGTAA-3′), designed by Scarabel et al. (2007) and Hada et al. (2021). PCR reactions were carried out in an Eppendorf Mastercycler thermal cycler, in 20 μl total volumes each of which containing 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. PCR conditions were 94 C for 5 min, followed by 36 cycles of 94 C for 5 min, 53 C and 58 C for GC-ALS-197 – AMA-2R and AMA-3F – GC-ALS574, respectively, for 40 sec, and 72 C for 40 sec, followed by a final extension step of $72^{o}C$ for 8 min. Successfully amplified PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey – Nagel, Germany) and were sequenced in both directions using the PCR primers in a 3730 automatic sequencer. Sequences were read, edited, and aligned in the software MEGA (Molecular Evolutionary Genetics Analysis) version 7 (Kumar et al. 2016) in comparison to *Arabidopsis thaliana ALS* gene (GenBank Accession Number: X51514) to score the genotypes based on several known *ALS* point mutations such as Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654 that have been previously associated with resistance to herbicides.

## Statistical Analysis

Fresh weight data (expressed as a percent reduction of the nontreated control) of the whole plant pot experiments used to evaluate the response of redroot pigweed populations to ALS inhibitors and to selective herbicides registered for postemergence applications in corn. More specifically, a combined over the two experiments ANOVA was conducted to test for treatment by experimental-run interaction for the 12 putative R and two S redroot pigweed population, using a $14 \times 4 \times 3$ split-plot factorial approach. The 14 field-selected redroot populations were the main plots and the four herbicides by three herbicide rates were considered as the sub-plots. In addition, the experiments evaluating the response of the same R and S redroot populations to five preemergence herbicides was conducted twice using three replicated pots for each herbicide treatment and nontreated control in a randomized complete block design. A combined ANOVA over two experiments was performed to evaluate the response of the 14 populations, using a 14 (populations) x 5 (herbicides) x 2 (herbicide rates) split-plot approach, where the populations were the main plots and the five herbicides by two rates the sub-plots. The data were analyzed over the two experiments because the homogeneity of variances checked by Barlett's test (Snedecor and Cochran 1989) indicated no significant departure from normality. Differences between means were compared at $P < 0.05$ using Fisher's protected Least Significant Difference (LSD) test.

## Results and discussion

### Whole plant assays to postemergence herbicides

The two susceptible (S1 and S2) reference redroot pigweed populations were most effectively controlled (100%) exposed to $1\times$, $2\times$, and $4\times$ field-rates of the ALS-inhibiting herbicides imazamox and tribenuron-methyl, the synthetic auxin dicamba and the triketone tembotrione (Figure 1). All herbicide treatments with the recommended label field-rates had excellent efficacy against the S1 and S2 redroot pigweed reference populations. Similarly, the population R5 proved susceptible to all tested postemergence herbicides (Figure 1). The survival of this population in the field was possibly due to a failed herbicide application by the farmer. On the other hand, imazamox failed to control 11 of the 12 putative-R redroot pigweed populations, indicating that field-evolved ALS-inhibitor resistance was selected in Clearfield® sunflower or soybean fields. The overall reduction of biomass for these populations across all doses of imazamox against the untreated control was 1% (populations

R2, R9), 2% (R12), 10% (R1, R6, R10), 12% (R3), 14% (R4, R8, R11), 15% (R7). More specifically, the recommended (1×) postemergence-applied rate of imazamox provided only 0-6% control of 11 putative-resistant populations, while control was not improved even with the application of 2× (0-19%) and 4× (3-26%) maximum field rates of this herbicide (Figure 1). Moreover, the same 11 putative R redroot pigweed populations were not effectively controlled with tribenuron-methyl applied in Express Sun® sunflower technology, which alternates with Clearfield® sunflower technology in sunflower crop fields. The overall reduction of biomass for the remaining populations across all doses of tribenuron-methyl against the untreated control was slighlty higher compared to imazamox but remained low, at 2% (population R12), 8% (R2), 9% (R11), 14% (R10), 15% (R4), 17% (R6), 21% (R8), 22% (R1, R7, R9) and 27% (R3). In particular, the recommended (1×), 2×, and 4× maximum field rates of tribenuron-methyl resulted in 0 to 14%, 0 to 26%, and 7 to 42% control of the 11 field-selected populations, respectively (Figure 1). The unsatisfactory control of the 11 putative-R redroot pigweed populations with imazamox and tribenuron-methyl applied at rates higher than the maximum recommended field rates, strongly supports the evolution of cross-resistance to ALS-inhibiting herbicides in these populations, which could be attributed to the increased selection pressure of these two herbicides repeatedly applied for many years in sunflower and soybean crop fields. Although the level of ALS-inhibitor resistance was not determined in the present study, most redroot pigweed populations were virtually unaffected when exposed to 4× rates of either imazamox or tribenuron-methyl in the whole plant pot experiments. Furthermore, their phenotypic response was supported by the detection of the ALS-inhibitor resistance endowing Trp574Leu substitution with a widespread and increasing occurrence among grass and broad-leaved resistant weed populations (Beckie and Tardif 2012; Heap 2024). The target-site resistance in these populations was probably selected independently as a result of the repeated application of the herbicides and the high reproductive capacity of redroot pigweed in monoculture production systems, which contributed to large population sizes that reinforced the probability of independent evolution of resistance (Délye et al. 2013). In contrast, non-target-site resistance is expected to result from recurrent selection imposed by reduced herbicide rates, which is not the case in our study, where the farmers used the recommended or even higher rates of imazamox and tribenuron-methyl. The limited implementation of crop rotation or herbicide alteration with different sites of action for effective weed control was probably responsible for the evolution of weed cross-resistance to postemergence applied ALS-inhibiting herbicides. Our findings agree with those reported by Nandula et al. (2020) who found the evolution of high cross-

resistance to the ALS-inhibiting-herbicides pyrithiobac, imazaquin, and trifloxysulfuron in both redroot pigweed and tall waterhemp (*Amaranthus tuberculatus*) populations. In addition, Scarabel et al. (2007) reported a field-selected redroot pigweed population highly cross-resistant especially to the imidazolinones imazethapyr and imazamox, and also the sulfonylureas thifensulfuron-methyl, oxasulfuron, and nicosulfuron. Moreover, a redroot pigweed population originating from soybean fields in China, with multiple resistance to both ALS- and PPO-inhibiting herbicides, was determined resistant to chemically dissimilar ALS inhibitors (imidazolinones, sulfonylureas, and triazolopyrimidines) (Wang et al. 2019), whereas selected redroot pigweed populations originating from cotton fields in Brazil were reported cross-resistant to the ALS-inhibitors trifloxysulfuron-sodium and pyrithiobac (Francischini et al. 2019).

Regarding postemergence applied alternative chemical options, the benzoic derivative dicamba (synthetic auxin) registered for annual and perennial broad-leaved weed control in corn fields and winter cereals provided strong suppression to excellent control of many ALS cross-resistant redroot pigweed populations evaluated in the present study. Therefore, it can be used in corn following either sunflower or soybean crops in crop rotation implemented schedules to mitigate field-evolved ALS resistance in redroot pigweed populations. In detail, dicamba recommended field rate ($1\times$) reduced fresh weight of seven (R1, R3, R4, R6, R7, R10, R11) and four (R2, R8, R9, R12) ALS cross-resistant populations by 93 to 100% and 73 to 78%, respectively, whereas excellent (100%) control was achieved with the application of $2\times$ and $4\times$ rates of this herbicide (Figure 1). Moreover, the fresh weight of nine (R2, R3, R4, R6, R7, R8, R9, R10, R12), and two (R1, R11) ALS cross-resistant redroot pigweed populations treated with the recommended ($1\times$) rate of the triketone tembotrione was reduced by 72 to 77%, and 92 to 100%, respectively. In contrast, the corresponding reduction with the two- and four-fold rate of this herbicide was 100% (Figure 1). The fluctuation from fair to excellent control observed in several redroot pigweed populations to the recommended rate of tembotrione and, to a lesser extent, dicamba, may be attributed to the standing genetic variation of the populations (Tranel 2021), and may well be an indication of early stages of resistance evolution to these sites of action.

***Whole plant assays to alternative preemergence herbicides***

All preemergence herbicides (*S*-metolachlor+terbuthylazine, isoxaflutole, aclonifen, mertibuzin, and pendimethalin) applied at the maximum recommended ($1\times$) field rate provided excellent control (95-100%) of the 11 populations with resistance to the ALS

inhibitors imazamox and tribenuron-methyl as well as the three susceptible ones (data not shown). The findings presented here agree with those reported by Scarabel et al. (2007) in that a highly cross-resistant redroot pigweed population to the imidazolinones imazethapyr and imazamox, and also the sulfonylureas thifensulfuron-methyl, oxasulfuron, and nicosulfuron was effectively controlled by herbicides with other sites of action (PS II, 4-HPPD and PPO inhibitors). In addition, as reported by Francischini et al. (2019), ALS cross-resistant and multiple resistant to PS II inhibitors redroot pigweed populations evolved from cotton fields in Brazil were most effectively controlled by the chloroacetamide *S*-metolachlor and the dinitroaniline trifluralin. However, as Storm et al. (2019) reported that waterhemp populations have evolved herbicide resistance to the VLCFA herbicides *S*-metolachlor, dimethenamid-P and acetochlor, rational use of herbicides has to be implemented to delay the evolution or mitigate already field-selected resistance and most importantly reduce the risk of evolution of multiple herbicide resistance in *Amaranthus* species populations (Milani et al. 2021a).

### *Amplification and sequencing of the ALS gene fragment*

The phenotypic response determined in the whole plant experiments was further supported by the molecular analysis of the ALS fragment, which revealed that the underlying mechanism of cross-resistance between tribenuron-methyl (sulfonylurea) and imazamox (imidazolinone) was target-site mediated. The alignment of the nucleotide sequences of the amplified *ALS* gene fragments from all redroot pigweed populations investigated, showed that the three plants of each of the 11 sequenced R populations contained a single point mutation leading to the replacement of a TGG codon with a TTG codon at the 574 site in domain B of the *ALS* gene (Figure 2). Among the five conserved domains of the *ALS* gene, A and B domains constitute the gene regions in which most of the point mutations conferring resistance to biocides have been detected (Scarabel et al. 2007). The Trp574Leu mutation identified in our study is known to confer broad resistance to all classes of ALS inhibitors (Bernasconi et al. 1995), whereas the respective plants of the three susceptible populations (S1, S2, R5) were found with the wild type Trp (TGG) genotype (Figures 2, 3). Regarding the 11 R populations molecularly examined, eight were homozygous and three were heterozygous for the Trp/Leu alleles, whereas all plants of the three susceptible populations were homozygous for the wild type Trp genotype (Figure 2). The presence of the same resistance-conferring point mutation in resistant individual plants of all R redroot pigweed populations indicates that the application of ALS inhibitors exerts a strong selection pressure on wild-type populations. In

addition, the lack of ALS mutations in the R5 population provided further evidence of its susceptibility, following its effective control by all postemergence applied rates of both ALS-inhibiting herbicides in the whole plant pot assays (Figure 3). All the remaining genetic loci examined for all populations were characterized by the presence of the wild-type allele. The same amino acid substitution (Trp574Leu) has been reported in ALS-inhibitor herbicide-resistant redroot pigweed (Scarabel et al. 2007), tall waterhemp (Milani et al. 2021a) and Palmer amaranth (*A. palmeri*) (Milani et al. 2021b) populations originating from field crops in Italy. Moreover, four target-site point mutations resulting in amino acid substitutions in residues Ala122, Pro197, Ala205, and Trp574 of the *ALS* gene conferred ALS-inhibitor resistance in field-selected redroot pigweed populations (Huang et al. 2016). Similarly, redroot pigweed populations evolved target-site mediated resistance to the imidazolinone imazethapyr due to Ala205Val, Ser653Thr and Trp574Leu (Chen et al. 2015) and due to Trp574Leu and Pro197His (Jones et al. 2023) amino acid substitutions. The Trp574Leu mutation is the primary mechanism of resistance to ALS inhibitors in *Amaranthus* species, although the accumulation of multiple mutations in one plant is also common (Singh et al. 2019). The Trp574Leu has been shown to confer high levels and broad-spectrum resistance to all classes of ALS-inhibiting herbicides in redroot pigweed (Cao et al. 2021; Nandula et al. 2020) and other weedy *Amaranthus* species, as well as other weed populations (Beckie and Tardif 2012; Heap 2024). Evolution of ALS resistance is in most cases target-site mediated due to amino acid substitutions revealed by sequence analysis (Murphy and Tranel 2019) and is less commonly attributed to NTSR mechanisms. Moreover, early NTSR usually confers low to moderate levels of resistance compared to target-site-based herbicide resistance because of the gradual build-up of resistant alleles, requiring several generations to accumulate in individual plants (Délye 2013). The target-site resistance reported here was probably derived by independent selection because of the repeated application of the herbicides in combination with the high reproductive capacity of redroot pigweed in monoculture production systems, which contributed to large population sizes that reinforced the probability of independent evolution of resistance (Délye et al. 2013). In contrast, non-target-site resistance is expected to result from recurrent selection imposed by reduced herbicide rates, which is not the case in our study, where the farmers used the recommended or even higher rates of imazamox and tribenuron-methyl. Furthermore, the fact that redroot pigweed is a highly self-pollinating weedy species and displays occasional outcrossing might explain the rapid increase in the initial frequency of the dominant resistance-endowing mutant allele(s) and why most samples studied at the molecular level of *ALS* nucleotide

sequences were homologous at the *ALS* locus (Scarabel et al. 2007). However, regardless of the rapid evolution of herbicide resistance, the trait conferring ALS resistance is not associated with a growth or ecological penalty in resistant redroot pigweed populations (Sibony and Rubin 2003). This was also confirmed by Wang et al. (2022) who found no significant differences in various growth traits (leaf number, plant height or dry weight) between the S and ALS R redroot populations harboring the Trp574Leu *ALS* mutant allele. The lack of detectable resistance adaptation cost regarding growth and reproduction in field-evolved ALS-resistant weed populations will allow frequency enrichment and fixation of herbicide resistance endowing mutant alleles.

Based on the above findings, the implementation of long-term, diversified, integrated weed management strategies (chemical, mechanical and cultural) is essential to reduce the frequency of repeated herbicide applications and alleviate the intense selection pressure imposed to *Amaranthus* species populations. The use of different herbicide sites of action in annual rotations, tank mixtures and sequential applications can effectively reduce the selection pressure of herbicides. Implementation of crop rotation, achievement of increased crop competitiveness via weed suppressive cultivar selection and increased crop density, adjustment of planting dates, proper irrigation and fertilization management can be tailored to impede weed emergence and growth, reduce weed competition, soil seedbank, and finally the risk for field-selected herbicide resistance (Norsworthy et al. 2012). Mechanical and even hand weeding whenever possible (adoption of zero-tolerance strategy), utilization of harvest weed seed control systems and cleaning of harvest and other agricultural machinery further contribute to minimize further spread of ALS-inhibitor herbicide resistance in *Amaranthus* species (Milani et al. 2021a). In conclusion, herbicide-resistant weed management should primarily focus on reduced herbicide dependency by exploiting multiple and effective agronomic practices in a long-term integrated weed management strategy that optimize their efficacy against weeds and result in profitable crop production.


**Practical implications**

This is the first report of target-site resistance in redroot pigweed to ALS-inhibiting herbicides, in Greece. Redroot pigweed is a noxious weed species commonly infesting all major summer crops in Greece, impacting considerably their yield. Our study verified the evolution of cross-resistance in 11 redroot pigweed populations in Northeastern Greece, to

the ALS-inhibiting herbicides imazamox and tribenuron-methyl, registered for post emergence applications in sunflower and soybean crops. The main reasons for the field-selection of resistance could be attributed to the lack of crop rotation and the repeated use of the two previously mentioned ALS inhibitors. The molecular mechanism endowing resistance to the ALS inhibitors was target-site mediated due to a Trp574Leu substitution at the *als* gene, encountered in the 11 redroot pigweed populations. This mutation confers high levels of cross resistance to all ALS inhibitors, thus creating significant challenges for the chemical control of weed populations with the particular substitution. The use of different sites of action is imperative for the control of such populations. Here, postemergence herbicide application with dicamba resulted in excellent control of most but not all populations, while tembotrione provided only fair control (70-80%) of most populations. Genetic variability among populations could partly explain the observed lower-than-expected efficacy with the labeled rates, mainly of tembotrione but also of dicamba on certain redroot pigweed populations. Furthermore, it cannot be ruled out that the sub-optimal levels of control are an indication of early stages of herbicide resistance evolution in the respective populations and sites of action. Such undesirable development would leave even fewer alternative options for postemergence chemical control of redroot pigweed populations with ALS resistance. Conversely, the preemergence herbicides *S*-metolachlor+terbuthylazine, isoxaflutole, aclonifen, metribuzin, and pendimethalin registered for use in sunflower, soybean, and corn, resulted in excellent control of all redroot pigweed populations. This work reinforces and broadens existing knowledge concerning selection of ALS-resistant redroot pigweed populations due to widespread and persistent use of ALS-inhibiting herbicides. For long-term redroot pigweed control it is essential to implement measures that reduce the risk of resistance selection and establishment, such as, adopting crop and herbicide rotation, increased crop density to suppress weed growth, selecting competitive crop varieties, using proper fertilization and irrigation schedules, applying mechanical and hand weeding, and by reducing spread of field-to-field or within field ALS-resistant populations.

**Competing Interests**

Competing interests: The authors declare none.

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**Table 1.** Geographical location of the studied redroot pigweed populations originating from arable field crops (R1-R12) and non-cultivated areas (S1, S2) at the prefecture of Kavala, north eastern Greece.

| Population | Area | Location |
|---|---|---|
| R1 | Eleftheroupoli | $40^o93047$ N, $24^o27185$ E |
| R2 | Eleftheroupoli | $40^o93011$ N, $24^o26304$ E |
| R3 | Eleftheroupoli | $40^o93870$ N, $24^o26849$ E |
| R4 | Hortokopi | $40^o93710$ N, $24^o26015$ E |
| R5 | Hortokopi | $40^o94253$ N, $24^o25323$ E |
| R6 | Hortokopi | $40^o95303$ N, $24^o26505$ E |
| R7 | Hortokopi | $40^o94880$ N, $24^o25559$ E |
| R8 | Hortokopi | $40^o95013$ N, $24^o25943$ E |
| R9 | Antifilippoi | $40^o95983$ N, $24^o24821$ E |
| R10 | Antifilippoi | $40^o96278$ N, $24^o24242$ E |
| R11 | Antifilippoi | $40^o96852$ N, $24^o23778$ E |
| R12 | Kokkinohoma | $40^o93847$ N, $24^o28743$ E |
| S1 | Eleftheroupoli-Hortokopi | $40^o95440$ N, $24^o26620$ E |
| S2 | Hortokopi | $40^o93766$ N, $24^o25149$ E |

**Table 2.** Postemergence herbicide product sources and rates used in the whole plant dose response experiments.

| Herbicide | Trade name | Form[a] | Rate | Manufacturer |
|---|---|---|---|---|
| | | | g ai ha$^{-1}$ | |
| Imazamox | Pulsar 4 | SL | **40** [c] | BASF Hellas, Athens, Greece |
| | | | 80 | |
| | | | 160 | |
| Tribenuron-methyl | Granstar 50 | SG | **18.7** | FMC Hellas, Athens, Greece |
| | | | 37.5 | |
| | | | 75 | |
| Tembotrione | Laudis 66 | OD | **99** | BayerCrop Science, Athens, Greece |
| | | | 198 | |
| | | | **396** | |
| Dicamba | Dicamba 48 | SL | **288** | Agrotechnica, Thessaloniki, Greece |
| | | | 576 | |
| | | | 1152 | |

[a] Abbreviations: SL, soluble liquid; SG, water-soluble granules; OD, oil dispersion

[b] Tribenuron was applied with the surfactant Trend® 90 SL (Corteva Agriscience, Athens, Greece) at 0.1% v/v;

imazamox was applied with the surfactant Dash® HC (BASF Hellas, Athens, Greece) at 0.4% v/v.

[c] Bold letters indicate label recommended rates of the herbicides.

**Table 3.** Preemergence herbicide product sources and rates used in the whole plant dose–response experiments.

| Herbicide | Trade name | Form [a] | Rate | Manufacturer |
|---|---|---|---|---|
| | | | g ai ha$^{-1}$ | |
| S-metolachlor+Terbuthylazine | Gardoprim Gold Plus | SE | **1250+750** [b] <br> 2500+1500 | Syngenta Hellas, Athens, Greece |
| Isoxaflutole | Merlin Flexx L | SC | **99** <br> 198 | BayerCrop Science, Athens, Greece |
| Aclonifen | Challenge 600 | SC | **2400** <br> 4800 | BayerCrop Science, Athens, Greece |
| Metribuzin | Sencor 600 | SC | **210** <br> 420 | BayerCrop Science, Athens, Greece |
| Pendimethalin | Stomp Aqua 455 | CS | **1365** <br> 2730 | BASF Hellas, Athens, Greece |

[a] Abbreviations: SE, suspoemulsion; SC, suspension concentrate; CS, capsule suspension; SL, soluble liquid.

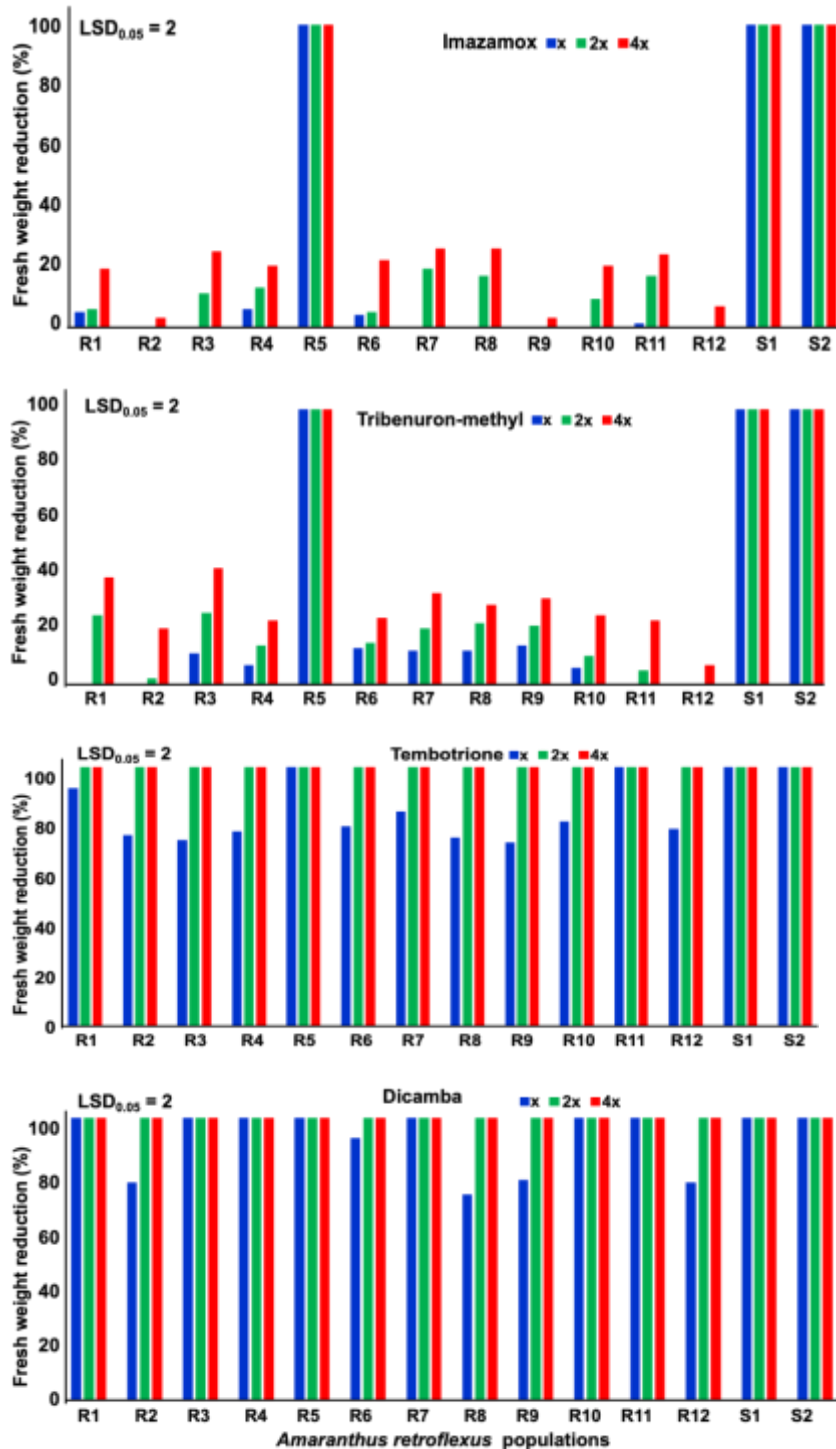[b] Bold letters indicate label recommended rates of the herbicides.

**Figure 1.** Fresh weight reduction (% of untreated control) of 14 redroot pigweed populations (12 putative resistant R1-12, and 2 reference susceptible S1, S2) by three rates of the herbicides imazamox, tribenuron, tembotrione and dicamba. Means were compared using Fisher's Least Significant Difference (LSD) test, at p<0.05.

```
X51514    TGGTTATGCAA TGG GAAGATCGGTT
S1        TGGTTATGCAA TGG GAAGATCGGTT
S2        TGGTTATGCAA TGG GAAGATCGGTT
R1        TGGTTATGCAA TTG GAAGATCGGTT
R2        TGGTTATGCAA TKG GAAGATCGGTT
R3        TGGTTATGCAA TTG GAAGATCGGTT
R4        TGGTTATGCAA TTG GAAGATCGGTT
R5        TGGTTATGCAA TGG GAAGATCGGTT
R6        TGGTTATGCAA TTG GAAGATCGGTT
R7        TGGTTATGCAA TTG GAAGATCGGTT
R8        TGGTTATGCAA TKG GAAGATCGGTT
R9        TGGTTATGCAA TTG GAAGATCGGTT
R10       TGGTTATGCAA TKG GAAGATCGGTT
R11       TGGTTATGCAA TTG GAAGATCGGTT
R12       TGGTTATGCAA TTG GAAGATCGGTT
```

**Figure 2.** Nucleotide sequence alignment of the *ALS* gene taken from plants of three susceptible (S1, S2, R5) and 11 R redroot pigweed populations using Molecular Evolutionary Genetics Analysis (MEGA7.0) software. The codons refer to the standard *Arabidopsis thaliana ALS* gene (GenBank: X51514) (first sample). The second, third and eighth samples represent the DNA sequences of the three susceptible redroot pigweed populations. The observed polymorphisms (point mutations) are highlighted in bold and correspond to the Trp574 position of the redroot pigweed *ALS* gene. IUPAC-IUB nucleotide codes: TGG (Trp), TTG (Leu), TKG [TGG/TTG (Trp/Leu)].
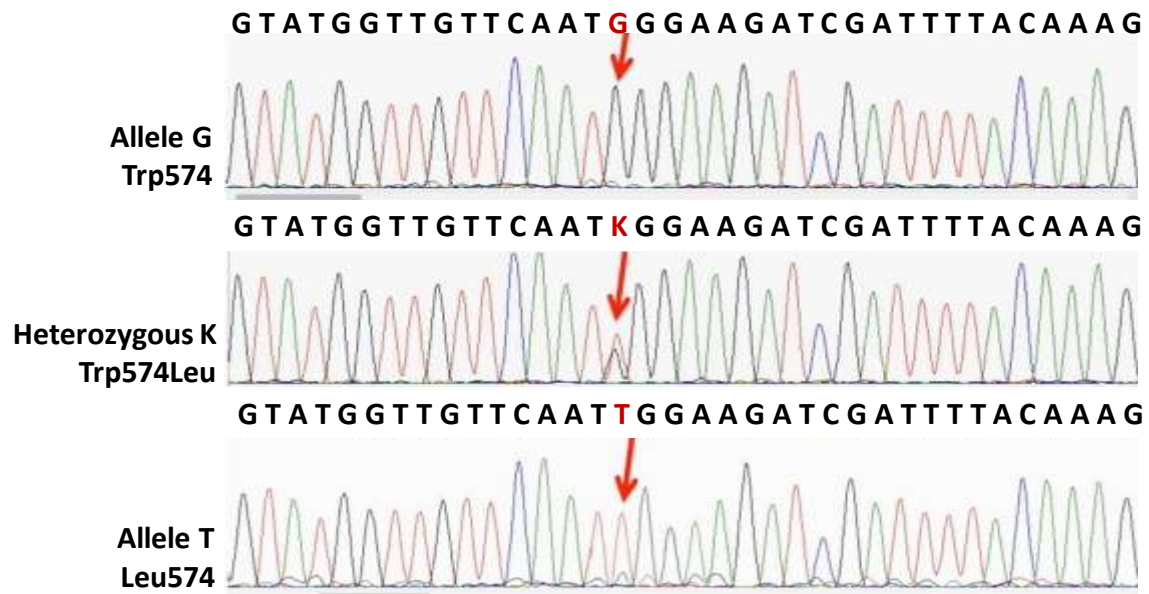
**ALS gene Trp574**



**Figure 3.** Point mutations were detected at codon 574 (indicated by red arrows) at chromatograms in the analyzed redroot pigweed samples. IUPAC-IUB nucleotide codes: TGG (Trp), TKG [TGG/TTG (Trp/Leu)], TTG (Leu).