



Several point mutations and metabolism confer cross-resistance to ALS-inhibiting herbicides in Tunisian wild mustard

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ABSTRACT

A growing number of weed biotypes showing resistance to acetolactate synthase (ALS)-inhibitors have been reported in several species, notably including *Sinapis arvensis* L. Two putative resistant (R) populations of *S. arvensis* from Tunisia were subjected to greenhouse and laboratory investigations to validate resistance to ALS-inhibitors and to determinate the mechanisms involved. The results indicated that both populations were resistant to four distinct ALS-inhibiting herbicides, tribenuron-methyl (TM), florasulam, flucarbazone and imazamox (IMZ), thereby confirming cross-resistance between them. The dose of (TM) required to achieve a 50 % reduction in plant growth (ED50) and 50 % mortality (LC50) in R populations of *S. arvensis* was found to be at least 60 times greater than the recommended field dose (18.7 g ai ha⁻¹) applied in cereal crops in Tunisia, indicating a significantly elevated resistance factor. Synergist experiments using malathion as a cytochrome P450 (Cyt-P450) inhibitor demonstrated a reduction in resistance to imazamox (IMZ) in both resistant (R) biotypes, indicating that Cyt-P450 plays a partial role in the resistance mechanism. In addition, ALS gene analysis identified three key point mutations, Pro197Ala, Asp376Glu and Trp574Leu, in both R populations. The docking analysis demonstrated that Asp376Glu mutation in *S. arvensis* could confer cross-resistance to IMZ and TM herbicides. CAPS and dCAPS methods were developed for detecting the Trp574Leu and Asp376Glu mutations, respectively, in *S. arvensis* and it was shown that work efficiently. Fortunately, the study also confirmed that 2,4-D still effectively controlled *S. arvensis* populations. This study provides valuable insights into the mechanisms underlying herbicide resistance in *S. arvensis* populations from Tunisia, demonstrating that both target-site resistance (TSR) and non-target-site resistance (NTSR) contribute to the species' broad-spectrum resistance against four dissimilar ALS-inhibitors.

1. Introduction

Wheat plays a vital role in global nutrition, it contributes 20 % of one's daily caloric and protein needed (Shiferaw et al., 2013). As the global population continues to grow rapidly, the demand for food escalates in tandem (FAO, 2012). Cultivated crops face adverse impacts from various ecological factors, stressors, climate-change and

plant-related threats, including insects, diseases, and weeds that lead to substantial reductions in crop yield (Ricroch et al., 2016).

Overall, weeds pose a significant challenge to wheat production and other crops, causing substantial yield losses (Jabran et al., 2015). Typically, weed management in wheat cultivation relies heavily on chemical herbicides, with the specific choice of herbicide dependent on the weed species. The widespread application of herbicides has led to an

Abbreviations: Wild mustard, *Sinapis arvensis*.

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increase in herbicide resistance, particularly when used improperly.

Sinapis arvensis L. (wild mustard) is a diploid ($2n = 18$), self-incompatible, annual weed species (Warwick et al., 2000) that is native to the Old World but has become widely introduced and naturalized across temperate regions globally. This species is characterized by a persistent seed bank, a highly competitive growth habit, and prolific seed production (Warwick et al., 2000), and has become a significant challenge for effective weed management. For instance, *S. arvensis* populations have developed resistance to widely used herbicides around the world, such as glyphosate, ALS-inhibitors and photosystem II (PSII) inhibitors among others (Goggin et al., 2019; Liu et al., 2021).

From a molecular standpoint, herbicide resistance mechanisms are categorized into target-site resistance (TSR) and non-target-site resistance (NTSR) (Torra and Alcántara-De Prado et al., 2022). Among the most frequently reported types of TSR mechanisms are point mutations, deletions in the target-site gene and altered expression (Torra and Alcántara-De Prado et al., 2022). In contrast, NTSR involves decreased herbicide uptake and translocation, vacuolar sequestration, a better capacity to control oxygen radicals, and enhanced metabolism mediated cytochrome P450s (Cyt.-P450s), glutathione S-transferases (GSTs), and glycosyltransferases (GTs) (Gaines et al., 2020). Resistance to ALS-inhibitors can result from both TSR and NTSR mechanisms, with TSR, primarily associated with mutations, considered the most prominent (Devine and Shukla, 2000). Until recently, the detection of these mutations has placed more emphasis on identifying TSR mechanisms compared to NTSR, even though they can coexist in the same population (Bai et al., 2019). However, the identification of plants lacking mutations in the ALS domain and surviving herbicide application has led researchers to focus on elucidating the NTSR mechanisms. Metabolism-based resistance to ALS-inhibitors has been identified in few dicotyledonous weeds (Veldhuis et al., 2000; Torra et al., 2021; Palma-Bautista et al., 2022a). In recent years, cases of resistance due to metabolism are increasing, mainly in grass weeds but also in broadleaf species, with genes associated with herbicide metabolism remaining largely unknown, particularly in dicots. Shen et al. (2022) demonstrated that a Cyt. P450 gene (*CYP77B34*) was responsible for tribenuron-methyl metabolism in *Brassicaceae* weed species.

Specifically, TSR develops as herbicides can no longer bind to their intended target sites due to mutations altering the amino acids in these locations. Key amino acid residues implicated in resistance to ALS-inhibitors include Ala122, Pro197, Ala205, Phe206, Asp376, Arg377, Trp574, Ser653 and Gly654 (Powles and Yu, 2010; Fang et al., 2022). Concerning *S. arvensis*, several cases of herbicide resistance have been documented globally (Heap, 2025). The first case was observed in 1993 in Ontario, Canada. Other countries such as Australia, Iran, Italy, Turkey, and the USA have reported resistance in *S. arvensis* biotypes, mainly in cereals (Heap, 2025). In *S. arvensis*, four distinct amino acid substitutions have been documented in the ALS gene, namely Thr122, Ser197, Glu376, and Leu574 (Warwick et al., 2005; Christoffers et al., 2006; Khaledi et al., 2019; Sin and Kadioglu, 2021). For instance, specific mutations in the ALS gene can lead to alterations in the shape and structure of its binding site, rendering herbicides less effective at inhibiting enzyme activity (Devine and Duke, 2014; Tardif et al., 2006). Ligand docking analyses play a crucial role in identifying these changes in binding affinity caused by resistance-conferring mutations (Devine and Duke, 2014; Tardif et al., 2006). Moreover, ligand docking studies can uncover instances of cross-resistance, where a single mutation in the ALS enzyme confers resistance to different families within an herbicide mode of action, and species-specific resistance, where different weed species develop resistance through unique ALS enzyme mutations (Devine and Duke, 2014; Tardif et al., 2006).

On the other hand, enhanced metabolism conferring resistance to ALS-inhibitors has been reported in several broadleaf weeds (Riar et al., 2012). Although the genes involved in metabolic resistance can vary depending on the weed species and history of herbicide application (Yu and Powles, 2014), most of these studies have predominantly identified

multiple Cyt. P450 genes that are either constitutively expressed or upregulated following ALS-inhibitor application (Liu et al., 2018). A study reported a metabolism-based resistance to ALS-inhibiting herbicides, namely, ethametsulfuron-methyl in *S. arvensis* (Veldhuis et al., 2000). The same study showed that metabolism in the resistance populations may be mediated, at least partially, by a Cyt-P450. Resistance to different auxins has been also reported in *S. arvensis* (Heap, 2025). A previous study observed no notable differences between resistant and susceptible biotypes in absorption, translocation, and metabolism, strongly suggesting that these factors do not directly contribute to the mechanism of auxinic herbicide resistance in *S. arvensis*. Instead, it was suggested that resistance might result from a change in the affinity of the auxin-receptor(s) for 2,4-D, as target-site resistance (TSR) mechanism (Peniuk et al., 1993).

Sinapis arvensis occurs in the north of Tunisia and is an important broadleaf weed species of arable field crops. Recently, farmers reported chemical failures in controlling *S. arvensis*, indicating reduction of herbicides efficacy. The aims of this study were: (1) to confirm and evaluate the resistance levels of two suspected *S. arvensis* populations collected from the northeastern region; and (2) to investigate the underlying resistance mechanisms, including potential TSR (mutations) and the role of enhanced metabolism as a non-target site resistance (NTSR) mechanism.

2. Material and methods

2.1. Source of plant materials

In summer 2019, six populations of *S. arvensis* were collected from wheat fields in Beja Northern Tunisia (Latitude: 36.7322° N Longitude: 9.1844° E). These fields had a crop rotation system encompassing cereal, legume, and recently, rapeseed which included both conventional and Clearfield® varieties. Within this rotational system, ALS-inhibitors were repeatedly applied. Specifically, during cereal years, TM was the predominant choice, whereas in rapeseed years with Clearfield® varieties, a combination of IMZ and metazachlor was favored. In contrast, conventional varieties received only metazachlor application. Additionally, quizalofop-P-ethyl and flumetsulam were utilized for weed management in some legume crops. Other modes of action were also employed to manage dicotyledonous weeds in addition to ALS-inhibiting herbicides. A preliminary screening was conducted on all populations with TM and IMZ described in section 2.3 at field rate (data not shown).

Among the six populations, two suspected resistant (R) populations (R37 and R38) of *S. arvensis* were selected for all the experiments. On the other hand, a susceptible reference population (S) was collected from roadside areas near the National Institute of Agronomy in Tunisia. This population had never been subjected to herbicide application.

2.2. Plant growth conditions

Seed germination was conducted in Petri dishes containing filter paper saturated with 5 mL of distilled water. Petri dishes were kept in the dark at 4 °C for 48 h (about two days), followed by an additional 24 h at room temperature. The seedlings produced were subsequently transplanted into plastic pots (7 cm × 7 cm × 9 cm) filled with a sand and peat mixture at a 1:1 (v/v) ratio and maintained under greenhouse conditions with a 16-h photoperiod, 300 μmol m⁻² s⁻¹ flux of photosynthetic photons, and approximately 75 % relative humidity. The plants were watered as necessary. Uniform plants were selected for whole-plant dose-response experiment.

2.3. Herbicide dose response

Once the plants had reached the four to six leaf stages (BBCH 14–16), both the resistant populations (R37 and R38) and the susceptible population (S) were sprayed with increasing doses of TM, florasulam,

flucarbazone, IMZ, 2,4-D (Table 1). The experiment followed a completely randomized design and was conducted twice, with each iteration comprising five replicates (two plants per pot) for every treatment and population combination. Plants that were not treated were used as controls. Application precision was upheld through the utilization of a precision bench sprayer featuring flat fan 110° nozzle tips (Hardi ISO LD-110-02). The system was operated at a pressure of 250 kPa and positioned at an elevation of 50 cm. Calibration procedures were rigorously implemented to ensure the delivery rate of 200 L ha⁻¹, thereby maintaining a methodologically sound and controlled experimental environment. Following 28 days of treatment, the plants were collected by cutting at ground level, and their fresh weight was assessed. The resulting fresh weight and survival data were transformed into percentage values for comparison with the untreated control plants.

2.4. Effect of malathion on tribenuron-methyl and imazamox resistance

An assay involving the application of a Cytochrome P450 (Cyt-P450) inhibitor, specifically malathion, was carried out on the three different populations of *S. arvensis*, selected as, S, R37, and R38. These plants were treated with malathion at a rate of 1000 g ai ha⁻¹, followed by a period of stabilization at ambient temperature for 1 h. Subsequently, TM and IMZ were applied independently in varying concentrations around the recommended field rate (R: 1x, 2x, 4x; S: 1/8x, 1/4x, 1/2x), (Table 2). For each treatment, defined as the combination of *S. arvensis* population and herbicide dose, five replicates were established. Each replicate corresponded to one pot containing two individual plants. Two control groups were established: one consisting of untreated plants and the other comprising plants treated solely with malathion. After 28 days, the fresh weights were recorded and analyzed to evaluate the reduction relative to the untreated control group. The experiment was repeated twice.

2.5. ALS gene sequencing

DNA was extracted from 10 plants from the S population as well as the resistant biotypes R37 and R38 using the Speedtools Plant DNA Extraction Kit (Biotools B&M Labs, Madrid, Spain). The study focused on specific regions of the *ALS* gene known to confer herbicide resistance, utilizing primer pairs P1/P2, SalF/SalR, and ALS3F/ALS3R which amplify regions containing the mutation points previously described. The study adhered to the protocol outlined by Palma-Bautista et al. (2022a) for the amplification and purification of *ALS* gene sequences, employing the BIOTOOLS DNA Purification Kit. The sequencing outcomes were analyzed by aligning and reviewing the sequences alongside the reference *S. arvensis* sequence from GenBank, accession number FJ655877, using the Geneious Prime software for visualization and comparison purposes (Kearse et al., 2012).

2.6. Development of dCAPS and CAPS markers for detection of Trp574Leu and Asp376Glu mutations, respectively

In order to realize a quick determination of the mutant Trp to Leu

Table 2

Different rates of tribenuron-methyl and imazamox applied to *Sinapis arvensis* populations, R37, R38 and susceptible (S) in the Malathion experiment.

Herbicide	Populations	Rates (g ai ha ⁻¹)		
		Low	Medium	High
tribenuron-methyl	R37, R38	18.7	37.4	74.8
	S	2.33	4.67	9.35
imazamox	R37, R38	40	80	160
	S	5	10	20

For R37 and R38: Low at the field rate, Medium at twice the field rate, and High at four times the field rate. For S: Low at one-eighth of the field rate, Medium at one-fourth, and High at one-half of the field rate.

amino acid change at 574 position of *ALS* in *S. arvensis*, a dCAPS primer was designed using dCAPS Finder 2.0. The primer *BsrI* (5'-CCAG-CATCTTGGCATGGTTATGCACT-3') was introduced by one forced mismatch (underlined) to create a restriction site for *BsrI* enzyme (Thermo Fisher, Catalog No. ER1251) in the wild biotype. Thus, the wild-type amplicons digested by *BsrI* would generate two bands of 280- and 40-bp. In contrast, the mutant-type amplicons would develop a single undigested 320-bp band due to the unavailability of the *BsrI* restriction site. To validate the reliability and precision of the dCAPS method, PCR was performed following the procedure described above, by using *ALSB* (Palma-Bautista et al., 2022a) as reverse primer.

The same was done for the change in position 376. The nucleotide substitution of Cat codon 376 results in an amino acid change from Asp to Glu, creates a *TaaI* restriction site. Therefore, the primer pair ARV1F and ARV1R (Palma-Bautista et al., 2022a) was used to amplify a 380-bp fragment, which was then digested with *TaaI*. Mutant resistant 376-Glu alleles would generate a single undigested 380 bp band, while wild-type 376-Asp alleles would generate a 320 bp band, plus and 60 bp fragment.

In both cases, after PCRs, 5 µg of PCR product was digested with 1 µL of the corresponding FastDigest restriction enzyme in a total volume of 30 µL according to the manufacturer's recommendations. The digested and undigested PCR amplicons were separated on 2 % agarose gels. The dCAPS Finder 2.0 software was employed to select optimal restriction enzymes for CAPS/dCAPS analysis (<http://helix.wustl.edu/dcaps>) (Neff et al., 2002). To validate the accuracy of the dCAPS/CAPS methods, 19 plants from the R populations and 5 plants from the S population were randomly selected for dCAPS analysis; in the case of the CAPS method 18 individual plants were selected, 6 S and 12 R. All these individual plants were sequenced to correlate results of dCAPS/CAPS with results of sequencing.

2.7. ALS enzyme modelling and ligand docking

The nucleotide sequence of *SarALS* (*S. arvensis* *ALS*) was retrieved from the NCBI database. A structural model of *SarALS* was developed using the SWISS-MODEL platform, employing the crystal structure of *Arabidopsis thaliana* *ALS* (AtALS) (PDB ID: 3E9Y) as the template. This template selection was consistent with its prior application in a structural study of *Sinapis alba* *ALS* (Chtourou et al., 2024). The structural

Table 1

Range of herbicide doses applied to *Sinapis arvensis* populations (R37, R38, and Susceptible S) in Dose-Response Experiment.

Herbicide	Commercial Product	Field rate g ai ha ⁻¹	Pop	Rates g ai ha ⁻¹
florasulam	Nikos® (5 % w/v)	7.5	R37, R38 S	0.23 0.46 0.93 1.875 3.75 7.5 15 30 0.23 0.46 0.93 1.875 3.75 7.5 15 30
flucarbazone	EVEREST® 70 % w/w	30	R37, R38 S	3.75 7.5 15 30 60 120 0.93 1.87 3.75 7.5 15 30
imazamox	Pulsar 40® 4 % w/v	40	R37, R38 S	1.25 2.5 5 10 20 40 80 160 0.62 1.25 2.5 5 10 20 40 80
tribenuron-methyl	Granstar SX® 50 % w/w	18.7	R37, R38 S	4.67 9.35 18.7 37.4 74.8 149.6 299.2 598.4 1196.8 0.29 0.58 1.16 2.33 4.67 9.35 18.7
2,4-D	Esteron 60® 60 % w/v	600	R37, R38 S	18.75 37.5 75 150 300 600 18.75 37.5 75 150 300 600

assessment of the SarALS model was also performed using the same server. To investigate the binding of herbicides to SarALS, TM and IMZ chemical structures, were prepared with MarvinSketch and 3D-modelled in Maestro (Schrödinger) to conduct *in silico* docking using Glide (Schrödinger). The number of top poses was set to 10 and the radius of pose clustering and other parameters were kept as default for each docking. Additionally, the structural model of SarALS was

minimized and prepared for docking using Maestro (Schrödinger). ChimeraX (UCSF ChimeraX: Tools for structure building and analysis) was used to visualize and analyze the docking results and structural models.

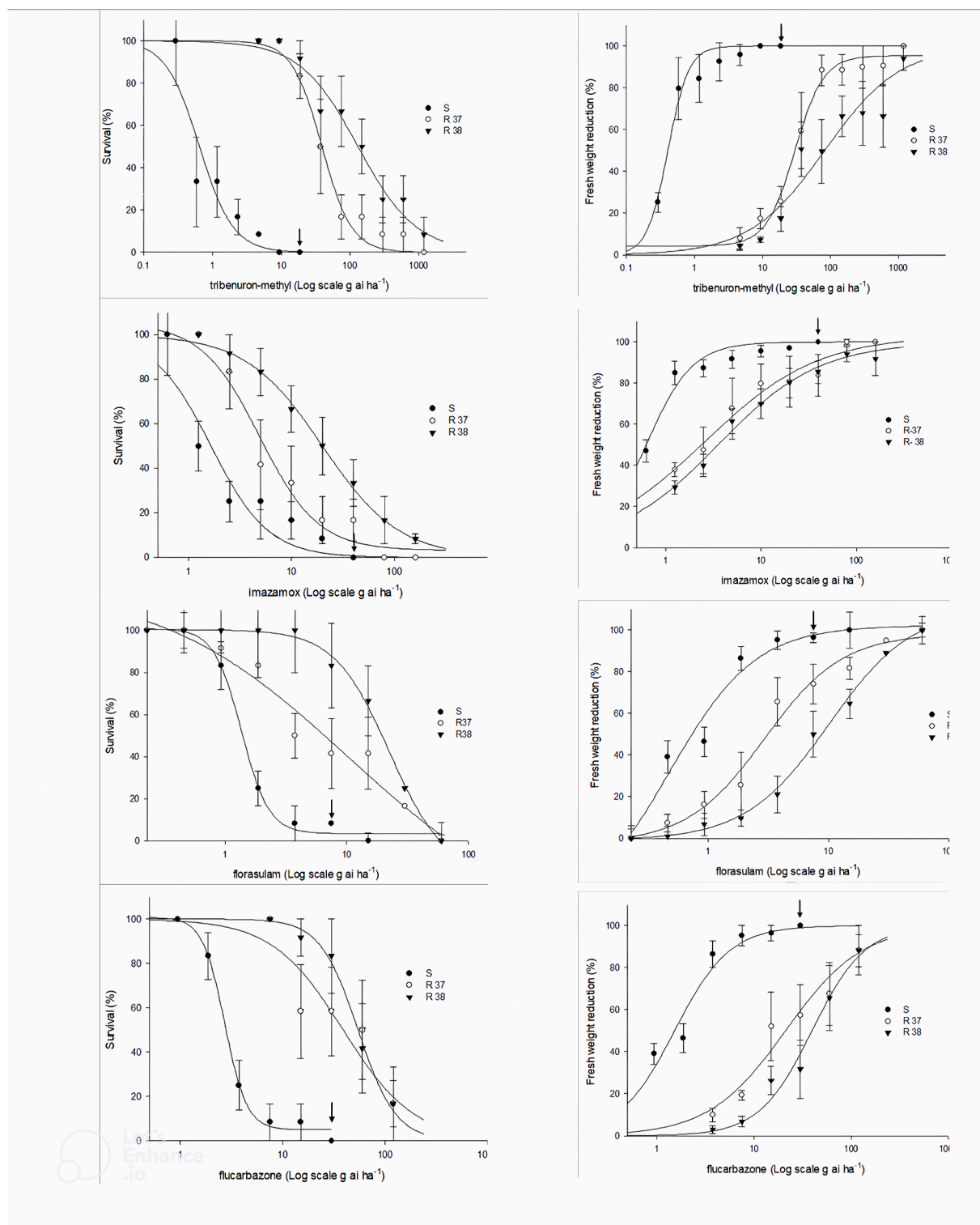


Fig. 1. Percent survival and fresh weight reduction relative to untreated controls of *Sinapis arvensis* populations (R37, R38, and susceptible (S)), treated with tribenuron-methyl, imazamox, florasulam, and flucarbazone, assessed 28 days after treatment. The arrow indicates the recommended field rate of the herbicides.

2.8. Data analyses

Analyses of dose-response data in the whole plant were performed using the Sigma Plot 12.0, a product of SPSS Inc. Chicago, IL, to implement a nonlinear log-logistic regression model. The purpose of this model was to determine the dosages of herbicide that would decrease plant growth by 50 % (ED₅₀) and lead to a 50 % mortality rate (LC₅₀) within the populations under study, using the untreated control as a reference. The resistance factor was calculated by comparing the ED₅₀ or LC₅₀ values of the resistant populations to those of the susceptible population (S). We applied the following formula for the log-logistic model in our research:

$$Y = c + [(d - c) / 1 + (x / g)^b]$$

In the formula, Y denotes the fresh aboveground biomass as a percentage of the control not treated. c and d are the lower and upper plateaus, b characterizes the curve's slope, and g signifies the herbicide dosage at which the graph's midpoint is located and represents either the ED₅₀ or LC₅₀ values. The herbicide dosage is the independent variable x. The Resistance Factor (RF) was calculated by dividing the determined ED₅₀ or LC₅₀ value of the resistant biotypes by that of the susceptible (S) population. The metabolism assay data for TM and IMZ were assessed with Tukey's statistical test to identify significant differences, considering a p-value below 0.05 to be statistically noteworthy.

3. Results

3.1. ALS-inhibitors dose-response experiment

Resistance to TM, IMZ, flucarbazone, and florasulam in the R37 and R38 populations was confirmed in this study, as showed by survival rates and the percentage of fresh weight reduction (Fig. 1). The whole-plant response experiments established that both R populations showed extremely high resistance levels to TM. In TM treated R37 and R38 plants, increasing herbicide rates did not significantly affect survival and fresh weight reduction (%). Compared to the S population, the TM LC₅₀ R37 and R38 were 39.06 and 131.24 g ai ha⁻¹, respectively, while the ED₅₀ were 30.03 and 82.60 g ai ha⁻¹, respectively. For the S population, the LC₅₀ and ED₅₀ were 0.64 and 0.40 g ai ha⁻¹, respectively. However, based on both survival and fresh weight reduction, the R38 population showed a higher resistance factor (202.56 and 203.35, respectively) compared to the R37 population (60.33 and 73.93, respectively) (Fig. 1, Tables 3 and 4). In the case of IMZ, the R38 population displayed greater resistance compared to R37, showing higher survival rates and lower fresh weight reduction. The LC₅₀ needed to manage 50 % of the R37 and

Table 3

Non-linear regression parameters estimated for the survival of *Sinapis arvensis* populations (R37, R38 and susceptible (S)) treated with tribenuron-methyl, imazamox, forasulam and flucarbazone.

Herbicide	Population	Slope	LC ₅₀ (g ai ha ⁻¹)	RF
tribenuron-methyl	S	-1.77 ± 0.52	0.64 ± 0.11	1
	R 37	-2.00 ± 0.30	39.06 ± 5.86	60.33
	R 38	-1.02 ± 0.11	131.24 ± 6.02	202.56
florasulam	S	-3.68 ± 0.54	1.36 ± 0.07	1
	R 37	-1.06 ± 0.53	10.17 ± 8.82	7.47
	R 38	-2.41 ± 0.25	22.41 ± 2.36	16.47
flucarbazone	S	-3.68 ± 0.47	2.85 ± 0.13	1
	R 37	-1.24 ± 0.30	38.87 ± 8.05	13.63
	R 38	-2.34 ± 0.18	54.37 ± 2.09	19.07
imazamox	S	-1.55 ± 0.41	1.64 ± 0.29	1
	R 37	-1.46 ± 0.26	5.75 ± 0.78	3.48
	R 38	-1.16 ± 0.05	20 ± 0.86	12.12

RF is the resistance factor (LC₅₀ R/LC₅₀ S) and LC₅₀ is the effective dose needed for 50 % survival. The P values (the non-linear model's probability threshold of significance) for all non-linear regressions fall between <0.0001 and 0.0029.

Table 4

Non-linear regression parameters estimated for the fresh weight reduction relative to untreated control of *Sinapis arvensis* populations (R37, R38 and susceptible (S)) treated with tribenuron-methyl, imazamox, florasulam and flucarbazone.

Herbicide	Population	Slope	ED ₅₀ (g ai ha ⁻¹)	RF
Tribenuron-methyl	S	2.85 ± 0.52	0.40 ± 0.02	1
	R 37	1.56 ± 0.20	30.03 ± 5.82	73.93
	R 38	0.78 ± 0.12	82.60 ± 9.24	203.35
florasulam	S	1.53 ± 0.64	0.47 ± 0.42	1
	R 37	1.06 ± 0.37	2.93 ± 0.60	6.23
	R 38	2.41 ± 0.20	10.08 ± 1.5	21.44
flucarbazone	S	1.53 ± 0.27	1.52 ± 0.17	1
	R 37	1.10 ± 0.16	21.12 ± 2.86	13.84
	R 38	1.59 ± 0.19	39.59 ± 3.38	25.95
imazamox	S	1.80 ± 0.33	0.62 ± 0.06	1
	R 37	0.80 ± 0.09	2.39 ± 0.31	3.82
	R 38	0.81 ± 0.04	3.55 ± 0.24	5.67

RF is the resistance Factor (ED₅₀ R/ED₅₀ S) and ED₅₀ is the effective dose needed for 50 % reduction in plant biomass. The P values (the non-linear model's probability threshold of significance) for all non-linear regressions fall between <0.0001 and 0.0012.

R38 populations were, respectively, 5.75 and 20 g ai ha⁻¹, respectively. However, the ED₅₀ was lower, 2.39 g ai ha⁻¹ for R37 and 3.55 g ai ha⁻¹ for R38. In contrast, the necessary dose for controlling the S population was very low than the field dose (Fig. 1, Tables 3 and 4). The resistance factor indicated higher resistance levels associated with flucarbazone herbicides. For R37, the LC₅₀ was 38.87 g ai ha⁻¹ and the ED₅₀ was 21.12 g ai ha⁻¹ presenting in this case similar resistance factor (13). Conversely, R38 presented higher LC₅₀ and ED₅₀, 54.37 and 39.59 g ai ha⁻¹, respectively, (Fig. 1, Tables 3 and 4). Regarding florasulam effects, the effective dose to achieve a 50 % reduction in growth for R38 was 10.08 g ai ha⁻¹, while for R37, it was markedly lower at 2.93 g ai ha⁻¹ as detailed in Fig. 1, Tables 3, and Table 4. Additionally, the lethal concentration to control half of the R38 population was 22.41 g ai ha⁻¹, which is significantly higher than that for the R37 population, which was 10.17 g ai ha⁻¹ refer to Fig. 1, Tables 4, and Table 5 for more details.

3.2. 2,4-D dose-response experiment

There was a negligible or even absent resistance for both populations (R37 and R38) compared to the susceptible S with resistant level (<2). The LC₅₀ needed to manage 50 % of the R37 and R38 *S. arvensis* populations were less than the suggested field dose of (600 g ai ha⁻¹) implemented in cereal crops across Tunisia, 123 and 115 g ai ha⁻¹, respectively (Table 5 and Fig. 2), while the ED₅₀ were 47.80 and 26.33 g ai ha⁻¹ (Table 6).

3.3. Potential metabolic resistance

Screening with malathion, a Cyt-P450 inhibitor, showed a decrease in fresh weight within the R populations. Table 7 illustrates quantitative differences in R populations when exposed to the herbicide alone versus the herbicide plus malathion. The impact of malathion was more pronounced with IMZ than with TM, mainly for R38 population of

Table 5

Non-linear regression parameters estimated for the survival of *Sinapis arvensis* populations (R37, R38 and susceptible (S)) treated with 2,4-D.

Population	Slope	LC ₅₀ (g ai ha ⁻¹)	RF
S	-1.96 ± 0.24	114.56 ± 8.18	1
R 37	-2.32 ± 0.15	123.39 ± 4.12	1.07
R 38	-1.54 ± 0.25	115.28 ± 13.44	1.006

RF is the resistance factor (LC₅₀ R/LC₅₀ S) and LC₅₀ is the effective dose needed for 50 % survival. The P values (the non-linear model's probability threshold of significance) for all non-linear regressions fall between <0.0001 and 0.0004.

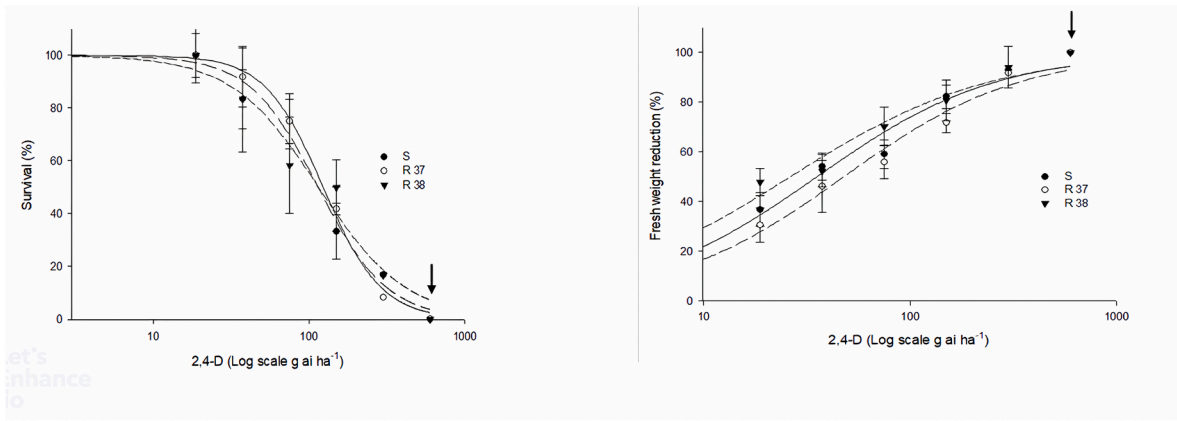


Fig. 2. Percent survival and fresh weight reduction relative to untreated controls of *Sinapis arvensis* populations (R37, R38, and susceptible (S)), treated with 2,4-D, assessed 28 days after treatment. The arrow indicates the recommended field rate of the herbicides.

Table 6
Non-linear regression parameters estimated for the fresh weight reduction relative to untreated control of *Sinapis arvensis* populations (R37, R38 and susceptible (S)) treated with 2,4-D.

Population	Slope	ED ₅₀ (g ai ha ⁻¹)	RF
S	1.00 ± 0.14	35.43 ± 4.49	1
R 37	1.02 ± 0.13	47.80 ± 5.57	1.34
R 38	0.90 ± 0.13	26.33 ± 3.87	0.74

RF is the resistance Factor (ED₅₀ R/ED₅₀ S) and ED₅₀ is the effective dose needed for 50 % reduction in plant biomass. The *P* values (the non-linear model's probability threshold of significance) for all non-linear regressions fall between 0.0005 and 0.001.

S. arvensis. At the highest rate of the herbicide, a significant difference between R38 plants treated only with TM and those treated with TM plus malathion was noted (Table 7). These findings suggest that metabolism can also contribute in the resistance to IMZ and TM herbicides in the R populations.

Table 7
Fresh weigh reduction in *Sinapis arvensis* populations R37, R38 and susceptible (S) treated with herbicides with and without malathion (M) at 1000 g ai ha⁻¹.

Herbicide	Population ^a	Treatment	Rates		
			Low	Medium	High
imazamox (IMZ)	S	IMZ	80 ± 3	84 ± 5	88 ± 4
		IMZ + M	79 ± 8 ns	80 ± 4 ns	90 ± 3 ns
	R37	IMZ	42 ± 9	57 ± 6	60 ± 4
		IMZ + M	65 ± 8 ns	79 ± 4 *	80 ± 4**
	R38	IMZ	38 ± 8	47 ± 5	60 ± 4
		IMZ + M	69 ± 6 **	73 ± 5 **	83 ± 4 *
tribenuron-methyl (TM)	S	TM	57 ± 7	65 ± 8	74 ± 5
		TM + M	59 ± 5 ns	61 ± 6 ns	72 ± 3 ns
	R37	TM	38 ± 5	48 ± 5	56 ± 6
		TM + M	42 ± 6 ns	55 ± 4 ns	73 ± 5 ns
	R38	TM	44 ± 5	64 ± 5	69 ± 6
		TM + M	54 ± 7 ns	72 ± 8 ns	88 ± 4 *

^aFor R37 and R38: Low at the field rate, Medium at twice the field rate, and High at four times the field rate. For S: Low at one-eighth of the field rate, Medium at one-fourth, and High at one-half of the field rate ns: non-significant difference.; * means significant difference (*p* < 0.05) and ** means significant difference (*p* < 0.001).

Codon	193	194	195	196	197	198	200	201	202	203	204
AA ^a	T	G	Q	V	P/ <u>S</u>	R	R	M	I	G	T
S	ACA	GGA	CAG	GTC	CCT	CGT	CGG	ATG	ATT	GGT	ACT
R37	ACA	GGA	CAG	GTC	TCT	CGT	CGG	ATG	ATT	GGT	ACT
R38	ACA	GGA	CAG	GTC	TCT	CGT	CGG	ATG	ATT	GGT	ACT
Similarity	***	***	***	***	**	***	***	***	***	***	***
Codon	371	373	374	375	376	377	378	379	380	381	382
AA ^a	V	R	F	D	D/ <u>E</u>	R	V	T	G	K	L
S	GTT	AGG	TTT	GAT	GAC	CGT	GTC	ACG	GGA	AAG	CTC
R37	GTT	AGG	TTT	GAT	GAA	CGT	GTC	ACG	GGA	AAG	CTC
R38	GTT	AGG	TTT	GAT	GAA	CGT	GTC	ACG	GGA	AAG	CTC
Similarity	***	***	***	***	**	***	***	***	***	***	***
Codon	570	571	572	573	574	575	576	577	578	579	560
AA ^a	M	V	M	Q	W/ <u>L</u>	E	D	R	F	y	k
S	ATG	GTT	ATG	CAA	TGG	GAA	GAT	CGA	TTC	TAC	AAA
R37	ATG	GTT	ATG	CAA	TTG	GAA	GAT	CGA	TTC	TAC	AAA
R38	ATG	GTT	ATG	CAA	TTG	GAA	GAT	CGA	TTC	TAC	AAA
Similarity	***	***	***	***	**	***	***	***	***	***	***

Codon	193	194	195	196	197	198	200	201	202	203	204
AA ^a	T	G	Q	V	P/ <u>S</u>	R	R	M	I	G	T
S	ACA	GGA	CAG	GTC	CCT	CGT	CGG	ATG	ATT	GGT	ACT
R37	ACA	GGA	CAG	GTC	<u>TCT</u>	CGT	CGG	ATG	ATT	GGT	ACT
R38	ACA	GGA	CAG	GTC	<u>TCT</u>	CGT	CGG	ATG	ATT	GGT	ACT
Similarity	***	***	***	***	**	***	***	***	***	***	***
Codon	371	373	374	375	376	377	378	379	380	381	382
AA ^a	V	R	F	D	D/ <u>E</u>	R	V	T	G	K	L
S	GTT	AGG	TTT	GAT	GAC	CGT	GTC	ACG	GGA	AAG	CTC
R37	GTT	AGG	TTT	GAT	<u>GAA</u>	CGT	GTC	ACG	GGA	AAG	CTC
R38	GTT	AGG	TTT	GAT	<u>GAA</u>	CGT	GTC	ACG	GGA	AAG	CTC
Similarity	***	***	***	***	**	***	***	***	***	***	***
Codon	570	571	572	573	574	575	576	577	578	579	560
AA ^a	M	V	M	Q	W/ <u>L</u>	E	D	R	F	y	k
S	ATG	GTT	ATG	CAA	TGG	GAA	GAT	CGA	TTC	TAC	AAA
R37	ATG	GTT	ATG	CAA	<u>TTG</u>	GAA	GAT	CGA	TTC	TAC	AAA
R38	ATG	GTT	ATG	CAA	<u>TTG</u>	GAA	GAT	CGA	TTC	TAC	AAA
Similarity	***	***	***	***	* *	***	***	***	***	***	***

Fig. 3. Alignment of *ALS* gene sequence of plants from *Sinapis arvensis* populations (R37, R38 and Susceptible (S)). Positions refer to the known *ALS* gene sequence of *Arabidopsis thaliana*. Amino acid positions are indicated by *ALS* gene from *Arabidopsis thaliana*. Nucleotide base is indicated by A = adenine, C = cytosine, G = guanine, T = thymine. ^a Amino acid nomenclature. Red color refers to the amino acid substitution. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 8

Number of plants with alleles identified at positions 197, 376 and 574 in three different *S. arvensis* populations (two resistant, one susceptible, S). 10 plants were sequenced per population but some showed two distinct alleles in resistant populations.

Mutation position	AA ^a	Allele	R37	R38	S
574	W/L	Homozygote	0	1	–
		Heterozygote	2	3	–
376	D/E	Homozygote	1	1	–
		Heterozygote	4	1	–
197	P/S	Homozygote	4	4	–
		Heterozygote	0	0	–
					–
197 + 376	P/S + D/E	Homozygote (at positions 197 and 376)	0	1	–
197 + 376	P/S + D/E	Heterozygote (at position 376)	2	0	–
376 + 574	D/E + W/L	Heterozygote(at positions 376 and 574)	2	1	–
No mutation			3	1	10

^a Amino acid nomenclature.

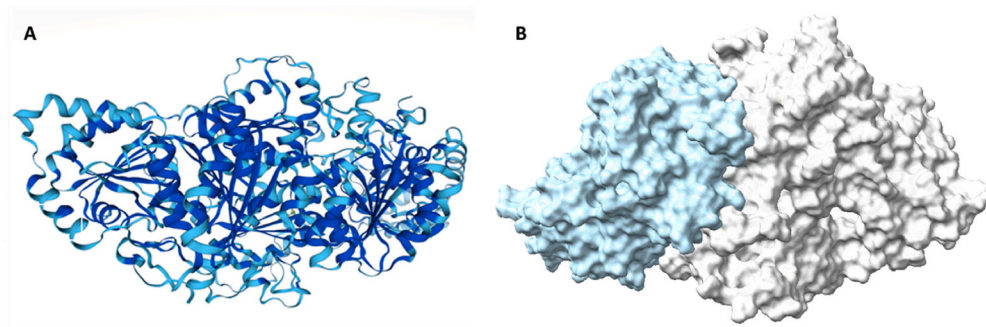


Fig. 4. *Sinapis arvensis* ALS structural model. Structural alignment between *Arabidopsis thaliana* ALS crystal structure (AtALS, PDB 3E9Y) in dark blue and the *Sinapis arvensis* ALS (SarALS) structural model in light blue (A). Surface of the dimeric SarALS model showing the surface of each monomer in light blue and light gray respectively (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Development of dCAPS and CAPS markers for detection of Trp574Leu and Asp376Glu mutations, respectively

The dCAPS/CAPS assays realized a quick determination of the allelic variation at the specific mutation site (574 and 376, respectively). The primer pair ALS3F and BsrI amplified a 320 bp DNA fragment. After restriction with BsrI, the wild type plants displayed a 280 bp digested band (with a 40 bp fragment invisible on the gel), while the homozygous mutant type plants showed a 320 bp undigested. Heterozygous plants displayed both the wild 280 bp and mutated 320 bp DNA fragments (Supplemental, Figs. S1 and S2). A total of 24 individual plants (5 S/10 R37/9 R38) were genotyped using the dCAPS method. Six of the nineteen showed digest mutant-type nucleotide/bands and wild-type nucleotides/bands, indicating heterozygous resistance at the position 574. In the same way, in the case of ARV1f-ARV1R amplification, after

restriction with *TaaI* the wild type plants displayed a 320 bp digested band (with a 60 bp fragment invisible on the gel), while the homozygous mutant type plants showed a 380 bp undigested. Heterozygous plants displayed both the wild 320 bp and mutated 380 bp DNA fragments (Supplemental, Fig. S3). Results from the CAPS method showed that from 18 individual plants (6 S/6 R37/6 R38) genotyped, 5 showed heterozygous resistance at position 376. These results confirmed the accuracy of both the dCAPS as the CAPS methods, for the Trp574Leu and Asp376Glu mutations, respectively, in the *S. arvensis* R populations.

3.6. Ligand docking

The amino acid sequences of AtALS and SarALS are highly conserved (Supplemental, Fig. S4). To investigate if the amino acid conservation was also maintained at the structural level, we modelled the protein

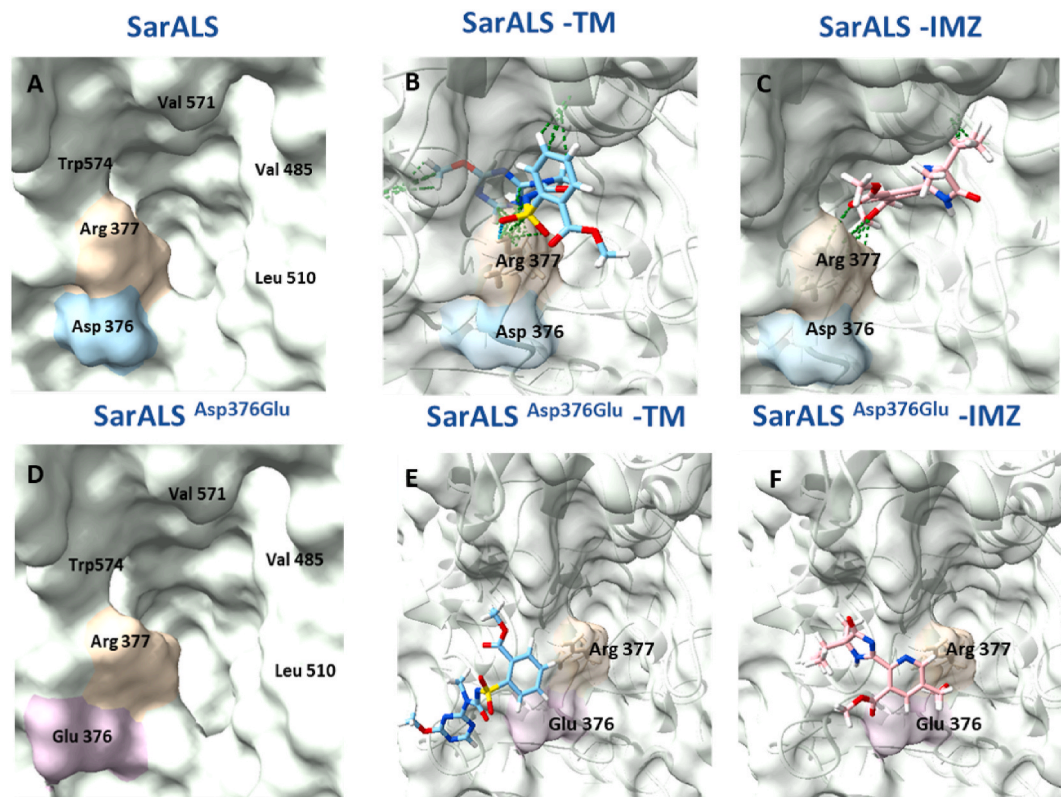


Fig. 5. Comparative analysis of the herbicide binding pocket within predicted structures for the *Sinapis arvensis* ALS enzyme (SarALS). Panels A–C depict: (A) SarALS active site structure, (B) Docking prediction of tribenuron-methyl (TM) (blue) bound to SarALS and (C) Docking prediction of imazamox (IMZ) (Pink) bound to SarALS. (D) SarALS^{Asp376Glu} active site. Panels (E) and (F) show no binding and unproductive interaction between TM and IMZ with the SarALS^{Asp376Glu} pocket, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

structure of SarALS using AtALS crystal structure as template. As expected, both structures show a high degree of conservation (Fig. 4A). Upon structural alignment using matchmaker, the SarALS model and the AtALS experimental structure highlighted structural similarities and showed a high degree of homology (93.39 %) (Supplemental, Table S1). The alignment, taking into account 544 pruned atoms has a RMSD value of 0.27 Å indicating a very high similarity between the positions of the Cα of both structures, with a significant overlap, particularly on the catalytic core (Fig. 4A). We utilized Glide docking to study the binding of sulfonylurea TM and imidazolinone IMZ herbicides to the modelled SarALS structure. By comparing the apo-SarALS structure to herbicide-bound SarALS docking results (Fig. 5), we confirmed the conserved binding mechanism among plant ALS enzymes, as reported by Garcia et al. (2017). The position of the different residues composing the binding pocket is well conserved (Lonhienne et al., 2022) as shown in Fig. 5. The binding mode of TM and IMZ to SarALS is the same as in other plant ALS. The results shows that TM binds to the conserved amino acid residues, Arg377, Gly121 and Trp574 while IMZ binding relies mostly on the interaction with Arg377 and Gly121. Molecular docking analysis using SarALS^{Asp376Glu} found in the R populations, confirmed that Asp376Glu, can alter the interactions between TM and IMZ and the active site of the enzyme, leading to a weaker binding affinity between the mutant ALS enzyme and herbicides. The SarALS form showed a docking scores of −2.703 with TM and −5.787 with IMZ, whereas the SarALS^{Asp376Glu} mutation showed a slight reduction in binding affinity, with docking scores of −2.622 with TM and −4.803 with IMZ, respectively (Supplemental, Table S2). The rearrangement of the pocket in SarALS^{Asp376Glu} leads to the loss of interactions with both TM and IMZ, including the H-bond between the herbicides and Arg377 in the pocket (Fig. 5). Also, in the case of TM, the crucial pi-pi interaction of the herbicide with the W574 of SarALS is lost. Consequently, neither TM nor IMZ can establish a productive binding pose in the SarALS^{Asp376Glu} protein (Supplemental, Figs. S5 and S6).

4. Discussion

In this study, we have identified the existence of ALS-inhibiting herbicides resistance in *S. arvensis* populations from Tunisia. The two studied populations showed resistance to distinct families of ALS-inhibitors and strongly suggested the presence of cross-resistance mechanisms. Both R populations responded similarly to the four ALS-inhibiting herbicides tested, only with slight differences in the levels of resistance. In this study, the dose–response experiments followed HRAC guidelines, using a full range of herbicide concentrations from sub-lethal to lethal levels (HRAC, 2025), consistent with earlier studies such as those conducted on *Sinapis alba* (Chtourou et al., 2024). Among the herbicides tested, tribenuron-methyl showed the highest resistance factor, with no complete fresh weight reduction even at 64 times the recommended field rate, confirming a high resistance level in alignment with previous reports (Chtourou et al., 2024). In contrast, although populations R37 and R38 were classified as resistant to imazamox based on their ED₅₀ and LC₅₀ values, these values remained low in absolute terms: only 6–9 % and 14–20 % of the recommended field rate were required to reduce fresh weight and cause 50 % mortality, respectively. From a practical agronomic perspective, this suggests that, despite meeting the criteria for resistance classification, these populations may still be effectively controlled under conventional field conditions.

The findings of this study contribute to the understanding of TSR in *S. arvensis*. They elucidate the impact of three key point mutations Pro197Ser, Asp376Glu, and Trp574Leu within the context of the 29 amino acid substitutions identified to date (Heap, 2025). These mutations were previously identified in other weed species, including *S. arvensis* (Tranel et al., 2017; Gaines et al., 2020; Palma-Bautista et al., 2022a). Similar frequencies of amino acid substitutions were observed between the two R populations, which is aligned with previous studies (Tranel et al., 2022). The target-site mutation at Pro197 is generally

considered to confer a high resistance level to sulfonylureas (SUs) herbicides, and usually low or no cross-resistance to imidazolinones (IMIs) herbicides (Devine and Shukla, 2000). A previous study on Canadian biotypes of *S. arvensis* demonstrated that this association was evident for the Pro to Ser substitution (Warwick et al., 2005), while Asp376Glu and Trp574Leu confer resistance across all ALS herbicide groups (Cruz-Hipolito et al., 2013). The mutation at position 574 caused higher resistance levels to IMI (IMZ) and SU (TM), as showed in previous studies (Tranel and Wright, 2002; Ntoanidou et al., 2019). Furthermore, Asp376Glu imparts notably heightened resistance to TM compared to other ALS gene mutations observed in various weed species (Zhang et al., 2017; Duhoux et al., 2015).

This study showed that in some individuals, all mutated sites presented a double or a single signal peak, indicating that the ALS gene double mutation could be in this case homozygous or heterozygous. The presence of plants with two distinct R alleles can be influenced by various factors, such as, the crop rotation systems (Kreiner et al., 2022). A different mutation is selected each year, yet the effectiveness of a single year of rotation may not suffice to entirely eliminate a particular mutation. Even changing the chemical family of ALS-inhibiting herbicides from SU (cereal year) to IMI (i.e. Clearfield® rapeseed) is not enough to eradicate all resistant plants with the 197 mutation, because, as shown, enhanced metabolism can also be present. Therefore, even if the herbicide is changed, these plants may still survive, leading to the persistence of this mutation within populations. The following year mutations which confer broad resistance to ALS-inhibitors (574 and 376) could be selected, plants harboring several R alleles can cross pollinate, and finally render individuals possessing double mutations in the population, which support the idea that herbicide resistance evolves the accumulation of mutations and resistance mechanisms over time (Gaines et al., 2020).

Reduced ALS binding capacity for herbicides is the most important resistance mechanism and is primarily responsible for resistance to ALS-inhibiting herbicides (Yang et al., 2018). This study represents the first molecular docking analysis with the SarALS enzyme to investigate the specific effects of the Asp376Glu amino acid substitution on the binding interactions with TM and IMZ. We have recently performed the structural analysis of *Sinapis alba* ALS focused on Trp574Leu and Pro197Ser mutations found in resistant populations (Chtourou et al., 2024). Since *S. alba* and *S. arvensis* are closely related, we focused our analysis in this work towards the SarALS Asp376Glu mutation. The simulation of ligand-receptor interactions revealed distinct effects of the Asp376Glu mutation on the interactions between SarALS and the herbicides. The 3D structure of SarALS^{Asp376Glu} demonstrates that this mutation fulfills a dual function, acting as a critical anchor for herbicides within the enzyme and contributing significantly in defining the shape of the substrate access tunnel. The Asp376Glu mutation affected the relative position of Arg377 in the binding pocket also affecting Trp574. Arg377 is a crucial amino acid for herbicide binding and establishes H-bonds both with TM and IMZ. Notably, for TM, the interaction of the substituted aromatic ring with the binding pocket is compromised due to the lack of the hydrogen bond to Arg377. Docking results also suggest that the pocket rearrangement in SarALS^{Asp376Glu} affects the orientation of Trp574 impeding the pi-pi interaction with TM. Overall, TM binding is severely affected in SarALS^{Asp376Glu}. Further docking analysis demonstrated that IMZ also fails to bind to SarALS^{Asp376Glu}, contributing to broad ALS inhibiting herbicide resistance. In this case, the loss of the hydrogen bond between IMZ and Arg377 in SarALS^{Asp376Glu} binding pocket could explain the IMZ resistance found in the *S. arvensis* populations. This observation supports the notion that the Asp376Glu mutation in *S. arvensis* could confer cross-resistance to IMI and SU herbicides, aligning with a previous study (Yang et al., 2018). However, a recent study on other weed species demonstrated that the Asp376Glu mutation confers resistance to IMZ, albeit at a lower level compared to imazethapyr, as observed in *Amaranthus retroflexus* and *Sorghum halepense*. Docking studies indicated that IMZ retains inhibitory activity

through a chalcogen interaction, which is not present in imazethapyr, resulting in differential resistance levels. This finding supports the variability in cross-resistance patterns and underscores the importance of considering species-specific responses, even within the same chemical family of ALS-inhibiting herbicides (Porri et al., 2024).

The dCAPS and CAPS assays designed in this paper could quickly identify the homo/heterozygous status of the plants at Trp574 and Asp376 amino acid residues without the gene sequencing. On the other hand, it is important to highlight its potential application in *S. alba*, as it is worth mentioning that the sequence used is the same as that of *S. arvensis*. Preliminary assays confirmed that both methods could be applied to this species too (data not shown).

The study also demonstrates that the application of malathion led to a reduction in resistance to IMZ within populations R37 and R38, significant at particular rates. On the other hand, the reduction in resistance to TM was only significant at the highest rate in R38 biotype. All mutations detected in this study confer resistance to TM, while mutations in position 197 usually do not confer resistance to IMZ or very low (Devine and Shukla, 2000). Both populations harbored Pro197Ser in homozygosis, 4 out of 10 plants sequenced. This would explain why the synergism between malathion and IMZ was stronger and significant compared to TM. These findings suggest the potential involvement of Cyt-P450s in the observed herbicide resistance. Enhanced metabolism is considered one of the most complex NTSR mechanisms (De Prado et al., 2005; Gaines et al., 2020; Dimaano and Iwakami, 2021), largely attributed to the increased activity of Cyt-P450 enzymes, which significantly speed up herbicide metabolism (Jugulam and Shyam, 2019). This aligns with prior studies, such as those conducted in *S. alba* by Palma-Bautista et al. (2022a), which have substantiated the role of Cyt-P450 enzymes in herbicide breakdown. This transformation is a complex process, implicating the concerted action of both Cyt-P450 enzymes and GTs (Cruz-Hipolito et al., 2013; Palma-Bautista et al., 2022b). A study on individual *Papaver rhoeas* plants showed that TSR and NTSR coexist, with a pronounced emphasis on the latter (Rey-Caballero et al., 2017).

Our study found that 2,4-D still effectively controls *S. arvensis* populations in Tunisia. However, previous research has shown resistance in *S. arvensis* which is unrelated to herbicide uptake or metabolism (Jugulam et al., 2012). Recent studies on its close relative, *S. alba*, sourced from similar geographic areas, have shown potential in evolving resistance to dicamba and 2,4-D, particularly in regions where auxin-based herbicides are extensively used (Chtourou et al., 2024). Proactive consideration of these findings in the context of *S. arvensis* resistance is crucial for the long-term effectiveness of agricultural practices.

In essence, this study identifies the fourth documented case of herbicide resistance in a Tunisian weed species. The findings reveal that resistance in *S. arvensis* is mediated by Both TSR and NTSR mechanisms, which (together/or independent) confer broad resistance to all ALS inhibitors. TSR reveals three distinct altered positions, while NTSR implicates Cyt-P450s in ALS-inhibiting herbicides metabolism, further enriching our understanding of herbicide resistance mechanisms in this species. These populations showed resistance to four chemical families of ALS-inhibiting herbicides among five, indicating the adaptability and resilience of *S. arvensis* populations, which poses challenges for effective weed management. The study emphasized the role of enhanced metabolic processes in NTSR, adding complexity to ALS inhibitor resistance mechanisms. The research underscores the need to proactively anticipate the emergence of multiple resistances in the future for sustainable weed management practices for *S. arvensis*.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2025.110043>.

Data availability

Data will be made available on request.

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