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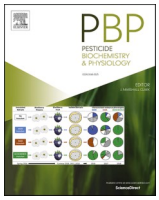

# Pro197Ser and the new Trp574Leu mutations together with enhanced metabolism contribute to cross-resistance to ALS inhibiting herbicides in *Sinapis alba*

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ABSTRACT

White mustard, (*Sinapis alba*), a problematic broadleaf weed in many Mediterranean countries in arable fields has been detected as resistant to tribenuron-methyl in Tunisia. Greenhouse and laboratory studies were conducted to characterize Target-Site Resistance (TSR) and the Non-Target Site Resistance (NTSR) mechanisms in two suspected white mustard biotypes. Herbicide dose-response experiments confirmed that the two *S. alba* biotypes were resistant to four dissimilar acetolactate synthase (ALS)-pinhibiting herbicide chemistries indicating the presence of cross-resistance mechanisms. The highest resistance factor (>144) was attributed to tribenuron-methyl herbicide and both R populations survived up to 64-fold the recommended field dose (18.7 g ai $ha^{-1}$). In this study, the metabolism experiments with malathion (a cytochrome P450 inhibitor) showed that malathion reduced resistance to tribenuron-methyl and imazamox in both populations, indicating that P450 may be involved in the resistance. Sequence analysis of the *ALS* gene detected target site mutations in the two R biotypes, with amino acid substitutions Trp574Leu, the first report for the species, and Pro197Ser. Molecular docking analysis showed that $ALS^{Pro197Ser}$ enzyme cannot properly bind to tribenuron-methyl's aromatic ring due to a reduction in the number of hydrogen bonds, while imazamox can still bind. However, Trp574Leu can weaken the binding affinity between the mutated ALS enzyme and both herbicides with the loss of crucial interactions. This investigation provides substantial evidence for the risk of evolving multiple resistance in *S. alba* to auxin herbicides while deciphering the TSR and NTSR mechanisms conferring cross resistance to ALS inhibitors.

## 1. Introduction

*Sinapis alba* L., commonly known as white mustard, is a member of the Brassicaceae family, which also contains oilseed crops, vegetables, and weeds (Warwick et al., 2005). Native from the Mediterranean area, white mustard causes problems in many countries, also in northeastern Tunisia. *S. alba* is a widespread and highly competitive weed mainly in wheat cropping system. It can cause reductions in wheat yield of up to 57% (Aricak and Bilgin, 2014), and in rapeseed up to 62% (Kumar and Singh, 2005). Traditionally, chemical control of dicotyledonous weeds in wheat includes the use of acetolactate synthase (ALS)-inhibiting herbicides, among other chemistries, with a significant increase in usage over the last few decades.

The ALS inhibitors specifically target the ALS enzyme, which plays a crucial role in the biosynthesis of the amino acids valine, leucine and isoleucine (Shaner, 1991; Duggleby et al., 2008). Presently, the inhibition of the ALS enzyme is achieved by six different herbicide families, which include sulfonylureas (SU), triazolopyrimidines (TP), imidazolinones (IMI), pyrimidinyl-thiobenzoates (PTb), sulfonanilides (SA) and triazolinones (SCT) (Heap, 2023). However, the persistent and repeated use of ALS herbicides has led to the development of resistance in various

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**Nomenclature**

White mustard
*Sinapis alba*

weed species. Worldwide, 170 weed species (105 dicots and 65 monocots) have been identified as resistant to ALS inhibiting herbicides (Heap, 2023). Herbicide resistance can be attributed to two primary mechanisms: Non-Target Site Resistance (NTSR) and Target-Site Resistance (TSR) (Gaines et al., 2020a). When it comes to ALS resistance, TSR mechanisms are commonly observed in broadleaf weeds (Yu and Powles, 2014a; Tranel et al., 2022a). On the other hand, NTSR mechanisms can hinder the herbicide's toxic form reaching the intended target site (Yu and Powles, 2014b; Hatami et al., 2016; Rigon et al., 2020). Enhanced metabolism is one of the most complex NTSR mechanisms, which involves the increased activity of cytochrome P450 monooxygenases (P450s), glutathione-S-transferases (GSTs), glucosyltransferases, and ATP-binding cassette transporters (De Prado et al., 2005; Jugulam and Shyam, 2019;Gaines et al., 2020a; Dimaano and Iwakami, 2021). Conversely, TSR, entails point mutations in genes encoding specific herbicide target proteins or overexpression of those genes (Yu and Powles, 2014a; Gaines et al., 2020b). Currently, over 24 weed species have demonstrated TSR mechanisms primarily attributed to mutations occurring at nine specific amino acid positions within the ALS *gene*. These positions are as follows: Ala-122 mutations have been observed in species such as *Alopecurus myosuroides*, *Descurainia sophia*, *Raphanus raphanistrum*, and *Sonchus oleraceus* (Tranel et al., 2017; Diez et al., 2019; Ovejero et al., 2020). Pro-197 mutations are commonly found in species including *Amaranthus* spp., *Echinochloa* spp., and *Lolium* spp. (Tranel et al., 2017; Gaines et al., 2020c; Cao et al., 2022). Ala-205 mutations have been identified in species such as *Amaranthus palmeri*, *E. crus-galli*, and *Eleusine indica* (Gaines et al., 2020c; Cao et al., 2022), while Phe-206 mutations have been observed in *Kochia scoparia*, *Solanum nigrum*, and *Xanthium strumarium* (Gaines et al., 2020c; Cao et al., 2022; Fang et al., 2022). Asp-376 mutations have been detected in species such as *Kochia scoparia*, *Lolium rigidum*, *S. alba, Sinapis arvensis* and *Conyza canadensis* (Gaines et al., 2020c; Cao et al., 2022), and Arg-377 mutations are present in *Amaranthus retroflexus*, *Chenopodium album*, and *C. canadensis* (Gaines et al., 2020c; Cao et al., 2022). Mutations at Trp-574 have been found in *Conyza bonariensis*, *Echinochloa colona*, and *L. multiflorum* (Gaines et al., 2020c; Cao et al., 2022; Palma-Bautista et al., 2022b), and Ser-653 mutations in *Echinochloa phyllopogon*, *Leptochloa chinensis*, and *Paspalum distichum* (Gaines et al., 2020a; Cao et al., 2022). Lastly, Gly-654 mutations have been identified in *E.crus-galli*, *L. rigidum*, and *R. raphanistrum* (Gaines et al., 2020c; Cao et al., 2022). Distinct amino acid changes can lead to varying levels of resistance. The level of resistance can differ based on the specific amino acid position that undergoes mutation and the resulting amino acid substitution (Tranel et al., 2022a). Research has demonstrated that certain amino acid changes in the ALS enzyme can confer resistance to specific groups of ALS-inhibiting herbicides. For instance, changes such as Ala1–22-Thr, Ala-205-Val, or Ser-653-Asn in the *ALS* gene are commonly associated with resistance to IMI herbicides. Conversely, Pro-197 mutations often confer high levels of resistance to SU herbicides. Moreover, mutations like Asp-376 or Trp-574 in *ALS* gene can provide broad-spectrum resistance encompassing all chemical groups that include ALS-inhibiting herbicides (Cao et al., 2022; Tranel et al., 2022b).

To date, two species of *Sinapis* genera that have developed resistance to ALS inhibitors have been identified worldwide. These species are *S. arvensis,* which has been reported in seven countries, including Australia, Canada, Iran, Italy, Spain, Turkey, and the United States, and *S. alba*, which has only been detected in two European countries, namely Cyprus and Spain. In *S. arvensis* multiple mutations have been identified in the *ALS* gene, namely Thr-122, Ser-197, Glu-376, and Leu-574. Conversely, in *S. alba*, up-to-date resistance to tribenuron-methyl (TM) is attributed to two amino-acid substitutions: proline by serine at position 197 (Warwick et al., 2005; Christoffers et al., 2006; Cruz-Hipolito et al., 2013; Khaledi et al., 2019; Sin and Kadıoglu, 2021), and aspartate (Asp) by glutamate (Glu) in position 376 in the *ALS* gene (Palma-Bautista et al., 2022b).

The agricultural system in Northeast Tunisia heavily relies on weather patterns, with crop production greatly influenced by temperature and precipitation. The region experiences mild and rainy winters, with temperatures ranging from 5 to 15 °C, while summers are hot and dry, with temperatures ranging from 30 to 45 °C and minimal rainfall. As a result, non-irrigated cereal cultivation is the only viable annual crop for farmers in this area. The typical crop rotation includes winter wheat or barley, legume crops and Clearfield® rapeseed, being a recent introduction in these cropping systems. These crops are typically sown in October and harvested in June of the following year, with wheat yields highly variable from year to year from 1 t $ha^{-1}$to 6 t $ha^{-1}$ (Rezgui and Fakhfakh, 2010) and rapeseed yielding approximately 1,27 t $ha^{-1}$ (National Institute of Large Crops (INGC), 2017). To control dicotyledonous weeds in cereal crops and rapeseed, TM and imazamox (Clearfield® varieties) are commonly used herbicides, followed by dicamba in wheat. However, in 2019, farmers in northeast Tunisia observed populations of *S. alba* that were not effectively managed by TM and imazamox, suggesting the ineffectiveness of these herbicides against this weed species. The objectives of this study were as follows: (1) to confirm and determine the level of the resistance to ALS inhibitors of suspected populations of *S. alba* collected from rapeseed fields in the northeast region of Tunisia; (2) to characterize the TSR mechanisms (mutation(s)) involved in the resistance to these herbicides; and (3) to investigate the presence of enhanced metabolism as NTSR mechanism.

## 2. Material and methods

### 2.1. Plant material

Seven populations were collected from rapeseed fields in Beja province, Northern Tunisia in summer 2019. These fields followed a winter wheat/barley/rapeseed rotation and had been treated with ALS inhibiting herbicides continuously for at least three years. The susceptible (S) reference population was collected from the roadsides of the National Institute of Agronomy of Tunisia and had never been subjected to any herbicide treatments. Additionally, another susceptible population from Spain was utilized as a standard/reference. Seeds were collected, scarified manually and stored at 4 °C until experiments. All populations had undergone a preliminary screening with TM, imazamox and dicamba described in section 2.2 at field rate (data not shown). Among the 7 populations, two suspected resistant (R) populations (R41, R29) of *S. alba* were selected for all the experiments.

### 2.2. Whole-plant dose-response experiment

The previously scarified seeds manually were germinated by placing them in Petri dishes with filter paper and 5 ml of distilled water. Dishes were then incubated in darkness at 4 °C for 48 h, followed by an additional 24 h at room temperature. Subsequently, the seedlings were transplanted into pots filled with a mixture of sand and peatmoss in a 1:1 (*v*/v) ratio. The pots were then placed in a greenhouse at 28/18 °C (day/night), with a photoperiod of 16 h, 300 μmol $m^{-2}$ $s^{-1}$ flux of photosynthetic photons, and ~ 75% relative humidity. Pots were watered as needed. For the whole plant greenhouse trials, the herbicides used were those listed in Supplementary I, Table S1. Applications were made using a precision bench sprayer equipped with flat fan 110° nozzle tips (Hardi ISO LD-110-02) at 250 kPa at 50 cm height, and calibrated to deliver 200 l ha − 1.At four to six leaf stages, the R (R29 and R41) and the S

populations were sprayed with TM, florasulam, imazamox, flucarbazone and 2,4-D at increasing doses (Table 1). The experiment was arranged as a completely randomized design and repeated twice with five repetitions (two plants per pots) per combination of treatment and population. Non-treated plants were used as controls. By the end of the experiment, the plants were harvested 28 days after treatment (DAT) at ground level and weighed to determine their fresh weight. Survival data were converted to percentages compared to untreated control plants.

### 2.3. Malathion effect

A screening trial using a (Cyt.) P450 inhibitor (malathion) was performed on the three *S. alba* populations. Plants from S-Tunisia, R29 and R41 were treated with malathion at 1000 g ai $ha^{-1}$ and then left for one hour at room temperature. Next, TM and imazamox were applied separately at three different doses around the field rate (R: 1×, 2×, 4×; S: ½x, ¼x, $^1/_8$x), and for dicamba a full dose response was applied, as detailed in Table 1. Since whole plant dose-response experiments (section 2.2) revealed no resistance or marginal resistance to 2,4-D, the malathion experiment was conducted exclusively with dicamba, as resistance was suspected from preliminary experiment (section 2.1). Two groups of controls were used: one group of non-treated plants and the second of plants treated only with malathion. Twenty-eight days after, the fresh weight was measured and analyzed to determine the reduction compared to the untreated control.

### 2.4. ALS gene sequencing

Target-site resistance investigation through point mutations was conducted on plants from S-Tunisia, R41, and R29 populations. DNA extraction was performed on 10 plants in the S population, and 20 plants each from the R41 and R29 populations. The Speedtools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Valle de Tobalina, Madrid, Spain) was used for this purpose. In this study, we employed a sequencing-based approach to examine the mutational profiles of plant populations treated with herbicides alone and in combination with malathion. To amplify the conserved domain regions of the *ALS* gene, three pairs of primers spanning all known mutation sites associated with ALS resistance were used: P1/P2 (containing Ala122, Pro197, Ala205, Phe206), SalF/SalR (Asp367, Arg377) and ALS3F/ALS3R (containing Trp574, Ser653, Gly654) (Palma-Bautista et al., 2022b). Gene fragments of ALS were amplified from each plant following conditions described in Palma-Bautista et al., 2022b. Gene fragments of *ALS* were amplified from each plant and then purified using the BIOTOOLS DNA Purification Kit. The sequencing results were visualized and aligned using Geneious Prime software, with the GeneBank accession FJ655877 of *S. arvensis* used as the consensus sequence for the alignment.

**Table 1**
Herbicides range of doses applied to *Sinapis alba* populations, R41, R29 and susceptible (S).

| Herbicide | Field rate g ai $ha^{-1}$ | Pop | Rates g ai $ha^{-1}$ |
|---|---|---|---|
| florasulam | 7.5 | R29, R41 | 0.23 0.46 0.93 1.875 3.75 7.5 15 30 |
| | | Tunisian S | 0.23 0.46 0.93 1.875 3.75 7.5 15 30 |
| flucarbazone | 30 | R29, R41 | 7.5 15 30 60,120 |
| | | Tunisian S | 0.46 0.93 1.87 3.75 7.5 15 30 |
| imazamox | 40 | R29, R41 | 1.25 2.5 5 10 20 40 80,160 |
| | | Tunisian S | 0.62 1.25 2.5 5 10 20 40 80 |
| tribenuron-methyl | 18.7 | R29, R41 | 4.67 9.35 18.7 37.4 74.8149.6299.2598.41196.8 |
| | | Tunisian S | 0.29 0.58 1.16 2.33 4.67 9.35 18.7 |
| | | Spanish S | 0.29 0.58 1.16 2.33 4.67 9.35 18.7 |
| 2,4-D | 600 | R29, R41 | 18.75 37.5 75,150,300,600 |
| | | Tunisian S | 18.75 37.5 75,150,300,600 |
| dicamba | 144 | R29, R41 | 9 18 36 72,144,288,576 |
| | | Tunisian S | 4.5 9 18 36 72,144 |

### 2.5. SaALS protein modelling and ligand docking

The nucleotide sequence of *SaALS* (*S. alba* ALS) was obtained from the NCBI database. To generate a structural model of SaALS, we utilized SWISS-MODEL using the crystal structure of *Arabidopsis thaliana* ALS (AtALS) (PDB 3E9Y) as template. The structural assessment of the SaALS model was also performed using the same server. To investigate the binding of herbicides TM and imazamox to SaALS, we modeled the structure of the chemical molecules and conducted in silico docking using Glide (Schrödinger). Additionally, different amino acid changes in SaALS were modeled using SWISS-MODEL and minimized with Maestro (Schrödinger). The docking results and structural models were visualized using Chimera X (UCSF ChimeraX: Tools for structure building and analysis).

### 2.6. Statistical analyses

The fresh-weight and survival data were fitted to a nonlinear log-logistic regression model using Sigma Plot 12.0 (SPSS Inc., Chicago, IL) statistical software, which allowed the estimation of herbicide dosages required to reduce plant growth by 50% (ED50) and to cause 50% mortality in a population (LC50) compared to the untreated control. The equation for the log-logistic model used in this study was as follows:

$$Y = c + [(d - c)/1 + x/g)\hat{}b].$$

where Y represents the survival or the fresh aboveground weight expressed as a percentage of the untreated control, c and d are coefficients corresponding to the lower and upper asymptotes, b is the slope of the response line, g is the herbicide dose at the point of inflection halfway between the upper and lower asymptotes (representing the LC50), and x (independent variable) is the herbicide dose. The resistance factor (RF) was calculated as the ratio of LC50 values for the R41 and R29 biotypes compared to the susceptible (S) biotype, using the equation:

$$RF = \text{LC50 of R}/\text{LC50 of S}$$

Data concerning metabolism assays for TM and imazamox were submitted to an analysis of variance (ANOVA) and Tukey's test was conducted to compare the means. The requirement of homogeneity of variance was verified. Differences with $p < 0.05$ were considered significant. Moreover, the LC50 values between S and R populations for auxins were compared using ANOVA. Finally, for dicamba metabolism experiments, the LC50 values between malathion treatments for each population were also compared using ANOVA.

## 3. Results

### 3.1. Dose-response assays

#### 3.1.1. Cross-resistance pattern to ALS inhibitors

The resistance factors and TM behavior of the two S populations (Spanish S and Tunisian S) were nearly identical: S-Spain had a resistance factor of 1, while S-Tunisia had a resistance value of 1.3. Conversely, based on survival (%) recorded in this study, both R41 and R29 populations demonstrated resistance to TM, imazamox, flucarbazone, and florasulam. The highest resistance factor (>144) was attributed to TM herbicide. The TM dose required to control the R *S. alba* populations by 50% (LC50) was higher than the field recommended dose

(18.7 g ai $ha^{-1}$) used in cereal crops in Tunisia, while for the S population this dose was below the field dose (10.69 g ai $ha^{-1}$). Based on the LC50 values, the R41 and R29 were at least 144 times more resistant than the S population. Thus, both R populations survived up to 64-fold the recommended field dose (Table 2 and Fig. 1) comparing to the two susceptible.

Regarding to imazamox behavior, the R29 and R41 populations showed the same LC50 (>160 g ai $ha^{-1}$); The LC50 needed to reduce 50% of the R *S. alba* population exceeded the recommended field dose of 40 g ai $ha^{-1}$ applied to cereal crops throughout Tunisia. In contrast, the necessary dose for controlling the S population was lower than the field dose. The R29 and R41 populations were at least 17 times resistant than the S population depending on the parameter estimated (Table 2 and Fig. 1). Lower resistance levels were attributed to flucarbazone herbicides as shown by the resistance index. Similar LC50 values (> 120 g ai $ha^{-1}$) were observed in plants from the two R populations of *S. alba* (Table 2). Furthermore, both R populations had a comparable survival reduction for florasulam; the corresponding LC50 values for R29 and R41 were > 30 g ai $ha^{-1}$ (Table 2 and Fig. 1).

#### 3.1.2. 2,4-D results

The R41, R29 and S *S. alba* populations had a general common behavior after the first days of 2,4-D application: appearance of epinasty, growth reduction and morphological damage at recommended doses (600 g ai $ha^{-1}$). Both R populations showed a significant distinct response to 2,4-D when compared to the S population (Table 3 and Fig. 2). However, resistant levels according to fresh weight reduction and survival were very low (≤ 2). The LC50 for R41 and R29 *S. alba* populations, were 53.02 and 60.05 g ai $ha^{-1}$, respectively.

### 3.2. Malathion effect

#### 3.2.1. ALS inhibitors

Screening using malathion as P450 inhibitor shown a reduction of fresh weigh in R populations. As shown in Table 4, R populations had differences when were exposed to the herbicide only and the herbicide plus malathion. With imazamox compared to TM, the impact of malathion was more evident, particularly on R41 population of *S. alba*. There is a discernible difference between plants treated with TM alone and TM plus malathion for R29 only at the highest rate (Table 4). These results indicate that metabolism is implicated in the resistance to TM and imazamox herbicide in the R populations.

#### 3.2.2. Dicamba

Regarding dicamba herbicide, there were minor differences between the S and the two R biotypes. However, only the population R41 presented a resistant factor >2. Screening using malathion as Cyt.P450 inhibitor showed a slight reduction of survival in the two R populations. As shown in Table 5 and Fig. 3, R41 and R29 had significant difference when were exposed to dicamba only and dicamba plus malathion. For R41, the LC50 decreased from 103.12 g ai $ha^{-1}$ without malathion to 36.66 g ai $ha^{-1}$ with the application of the P450 inhibitor. However, the S population did not suffer any effect from the application of malathion, observing that the LC50 values remained similar (Table 5 and Fig. 3).

### 3.3. SaALS gene sequencing assays

Comparison of *ALS* gene sequences in susceptible and resistant plants compared with that of *S. arvensis* (GenBank, accession FJ655877) revealed two non-synonymous mutations at positions 197, and 574 standardized to the ALS protein sequence of *Arabidopsis thaliana* and, which are already known to be involved in sensitivity to ALS-inhibiting herbicides in weeds.

At codon 197, the amino acid substitution Pro (CCT) to Ser (TCT) was detected only in resistant plants.

On the other hand, a single-nucleotide change occurred in amino acid position 574 (TGG) to (TTG) in the two resistant *S. alba* populations, giving the amino acid change Trp-Leu. These findings showed that most R plants sequenced contained at least one mutant-resistant allele (Table 6). No mutations were found in the rest of positions known to confer resistance to ALS inhibitors. As it is shown in Table 6, some of the R plants were heterozygous for those mutations, because overlapping peaks were observed in the chromatograms.

### 3.4. SaALS protein modelling and ligand docking

To better understand the molecular impact of the different mutations determined in our resistant populations to herbicide binding, we carried out a structural analysis of SaALS and tested herbicide binding through in silico docking. We utilized the SaALS amino acid sequence and the structure of AtALS (PDB 3E9Y) as template for protein modelling, which was performed using the SWISSMODEL structure homology-modelling server (https://swissmodel.expasy.org/). By structural alignment comparison of the SaALS model to the AtALS experimental structure, we observed a substantial overlap among each structure, specifically on the catalytic core, as expected form the high degree of homology of these two proteins at the amino acid level (Supplementary I, Fig. S1).

To simulate the binding of the sulfonylurea TM and imidazolinone imazamox herbicides to SaALS, we used Glide docking with our modeled SaALS structures. We compared the apo-SaALS structure to herbicide-bound SaALS docking results (Fig. 4). Analyzing the binding mechanism of each herbicide to SaALS, we could confirm that the binding mechanism is conserved among plant ALS enzymes (Garcia et al., 2017). TM and imazamox interact with residues Gly121 and Arg377 (Garcia

**Table 2**
Estimated parameters for non-linear regressions for survival for herbicides tribenuron-methyl, imazamox, forasulam and flucarbazone in *Sinapis alba* populations, R41, R29 and susceptible (S).

| HERBICIDE | POPULATION | SLOPE | LC50 | RF | $R^2$ | P |
|---|---|---|---|---|---|---|
| tribenuron-methyl | Spanish S | −1.54 ± 0.34 | 8.27 ± 6.82 | 1 | 0.986 | 0.0015 |
| | Tunisian S | −2.33 ± 0.60 | 10.69 ± 7.41 | 1.292 | 0.985 | 0.0001 |
| | R41 | −0.54 ± 0.12 | >1196.8 ± 2.49 | >144.7 | 0.836 | 0.3277 |
| | R29 | −11.16 ± 0.1 | >1196.8 ± 3.53 | >144.7 | 0.963 | <0.0001 |
| florasulam | Tunisian S | −2.163 ± 0.39 | 2.02 ± 7.32 | 1 | 0.991 | <0.0001 |
| | R41 | −7.50 ± 0.12 | >30 ± 6.03 | >14.883 | 1 | <0.0001 |
| | R29 | −3.42 ± 0.01 | >30 ± 1.8 | >14.883 | 1 | <0.0001 |
| flucarbazone | Tunisian S | −2.49 ± 1.02 | 13.07 ± 6.31 | 1 | 0.981 | <0.0001 |
| | R41 | −0.06 ± 0.01 | >120 ± 1.64 | >9.182 | 0.773 | <0.0001 |
| | R29 | −0.06 ± 0.01 | >120 ± 1.42 | >9.182 | 0.773 | <0.0001 |
| imazamox | Tunisian S | −1.67 ± 0.00 | 9.14 ± 0.00 | 1 | 1 | <0.0001 |
| | R41 | −1.038 ± 0.13 | >160 ± 2.53 | >17.7 | 0.962 | 0.0004 |
| | R29 | −11.09 ± 0.01 | >160 ± 3.93 | >17.7 | 0.004 | <0.0001 |

LC50 = effective dose required for 50% reduction in survival, RF = resistance Factor (LC50 R/LC50 S), $R^2$ = 1 – (sums of squares of the regression/corrected total sums of squares). *P* value = probability level of significance of the non-linear model.

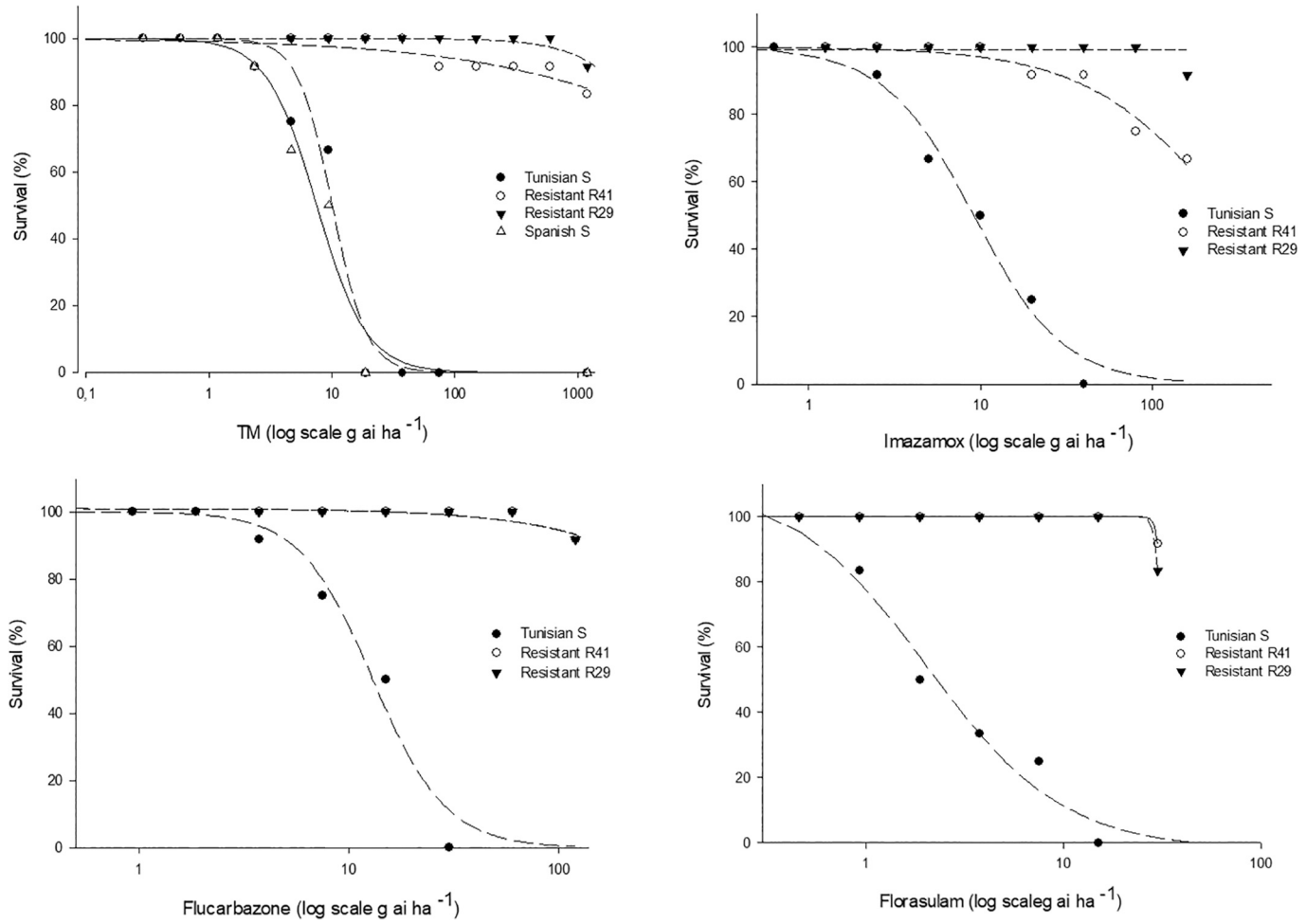


**Fig. 1.** Dose-response curves represented in percentages of survival for herbicides tribenuron-methyl (TM), imazamox, forasulam and flucarbazone in *Sinapis alba* populations, R41, R29 and susceptible (S).

**Table 3**
Estimated parameters (means ± standard errors) for survival for 2,4-D in *Sinapis alba* populations, R41, R29 and susceptible (S).

| POPULATION | SLOPE | LC50 | RF | $R^2$ | P |
|---|---|---|---|---|---|
| Tunisian S | −4.37 ± 1.23 | 30.13 ± 7.80 | 1 | 0.972 | <0.0001 |
| R41 | −2.82 ± 0.21 | 53.02 ± 3.52 * | 1.759 | 0.997 | <0.0001 |
| R29 | −3.36 ± 0.37 | 60.05 ± 2.56 * | 1.993 | 0.995 | <0.0001 |

LC50 = effective dose required for 50% reduction in survival, RF = resistance Factor (LC50 R/LC50 S), $R^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares). *P* value = probability level of significance of the non-linear model; *: denotes significant differences between S and resistant populations by ANOVA ($P < 0.05$).

et al., 2017) located at the ALS herbicide entering tunnel. The binding pose of TM also reveals a conserved mechanism, with much closer contact to amino acids in the active site tunnel than imazamox, including an important interaction between the aromatic ring of TM and Trp574 of SaALS, which is absent in the case of imazamox. Additionally, we also modeled two amino acid changes found in our herbicide resistant populations obtaining the predicted structures for $SaALS^{W574L}$ and $SaALS^{P197S}$. Trp574Leu produces a profound change in the herbicide binding pocket (Fig. 4). Trp574 is a very relevant aromatic amino acid for herbicide binding, stablishing π-π stacking contacts and hydrogen bonds with herbicide molecules. Therefore, the Trp574 Leu substitution weakens the binding affinity between the mutant ALS enzyme and herbicides due to the loss of crucial interactions, such as π-π stacking

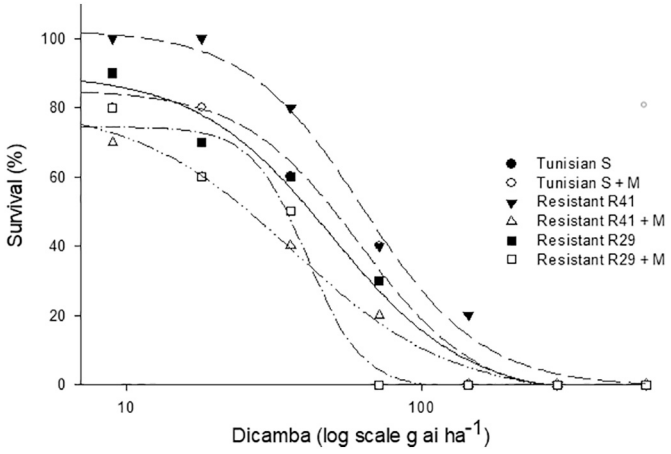


**Fig. 2.** Dose-response curves represented in percentages of survival for 2,4-D in *Sinapis alba* populations, R41, R29 and susceptible (S).

between the Trp574 and the herbicide. The loss of π-π stacking interactions is known to account for a 70-loss of affinity to the herbicide (Lonhienne et al., 2022). Additionally, due to the large rearrangement of the pocked in $SaALS^{W574L}$ (Fig. 4), other herbicide-target interactions are lost, including the hydrogen bond between the substituted ring of TM and R377 in the pocket. As a result, the mutated ALS enzyme has

**Table 4**
Fresh weight reduction in *Sinapis alba* populations, R41, R29 and susceptible (S) treated with herbicides with and without malathion (M) at 1000 g ai ha$^{-1}$.

| Herbicide | Malathion | RATE | S | R41 | R29 |
|---|---|---|---|---|---|
| | | Low | 55 ± 8 | 27 ± 3 | 10 ± 2 |
| | - (M) | Medium | 64 ± 6 | 50 ± 8 | 28 ± 9 |
| | | High | 72 ± 4 | 64 ± 5 | 38 ± 8 |
| | | Low | 56 ± 6 | 65 ± 5 | 19 ± 5 |
| imazamox | + (M) | Medium | 63 ± 3 | 73 ± 4 | 30 ± 4 |
| | | High | 70 ± 2 | 85 ± 6 | 45 ± 9 |
| | | Low | 0.9 ns | 0.0004** | 0.1 ns |
| | T.Test | Medium | 0.9 ns | 0.04 * | 0.7 ns |
| | | High | 0.8 ns | 0.03 * | 0.5 ns |
| | | Low | 54 ± 5 | 5 ± 3 | 20 ± 6 |
| | - (M) | Medium | 60 ± 3 | 22 ± 7 | 30 ± 3 |
| | | High | 71 ± 1 | 24 ± 9 | 27 ± 6 |
| | | Low | 59 ± 1 | 27 ± 8 | 21 ± 3 |
| tribenuron-methyl | +(M) | Medium | 60 ± 3 | 43 ± 5 | 40 ± 2 |
| | | High | 70 ± 9 | 24 ± 9 | 47 ± 1 |
| | | Low | 0.4 ns | 0.05 * | 0.8 ns |
| | T.Test | Medium | 0.9 ns | 0.2 ns | 0.08 ns |
| | | High | 0.9 ns | 0.1 ns | 0.01 * |

For R41, and R29: low = 1× field rate, Medium = 2× field rate, High = 4× field rate; For S: low = $^1/_8$x field rate, Medium = ¼x field rate, High = ½x field rate. ns: non-significant difference.; *: significant difference ($p < 0.05$); **: Very significant difference ($p < 0.001$); − (M) = herbicide only (without malathion); + (M) = with malathion plus herbicide.

reduced capacity to bind herbicides, leading to the emergence of herbicide resistance in plants harboring this amino acid change. The Pro197Ser amino acid change found in the resistant populations seems to have a different molecular mechanism for herbicide resistance. Although, the impact of this mutation in the pocket is reduced compared to the effect of W574L, the SaALS$^{P197S}$ change results in a local structural rearrangement that moves away several residues crucial for TM binding. As described previously, the interactions between the pyrrolidine ring of the Pro197 with the herbicide are lost in the Pro197Ser mutant (Lonhienne et al., 2022). However, this mutation is not expected to have a negative impact on imazamox binding because the interaction network of IMI herbicides is different from SU and does not rely so much in this amino acid. According to the docking results, imazamox can adopt a productive binding pose even in the SaALS$^{P197S}$ protein (Supplementary I, Fig. S2). However, the possible impact of this amino acid change on the accumulative inhibition exerted by IMI could not be discarded (Lonhienne et al., 2018; Lonhienne et al., 2022).

## 4. Discussion

Herbicide resistance in Tunisia has not been officially documented in several weed species, as per current knowledge. However, emerging studies have reported instances of resistance to ALS inhibitors in only two specific species, namely L. *rigidum* (Khammassi et al., 2019) and *Glebionis coronaria* (Hada et al., 2021). The findings of this study are particularly significant as they confirm, for the first time, the presence of *S. alba* populations with resistance to ALS inhibitors in Tunisia. In addition, the over-reliance with other herbicides has led to high risk of developing multiple resistance to auxin mimic and ALS inhibiting herbicides. The two populations tested were found to be resistant to four dissimilar ALS inhibiting herbicide chemistries, including IMI, SU, SCT and TP, indicating the presence of cross-resistance mechanisms.

The findings of this study offer significant contributions to our understanding of the molecular mechanisms underlying TSR resistance in *S. alba* populations to ALS inhibitors. Specifically, this study demonstrates the impact of two key point mutations, Pro197Ser and the newly discovered Trp574Leu in *S. alba*, on the binding of herbicides to the target enzyme SaALS. The Pro197Ser mutation has been previously reported in other weed species and has been associated with reduced herbicide binding affinity to the target enzyme (Powles and Yu, 2010; Yu and Powles, 2014a). Expanding upon this foundation, prior

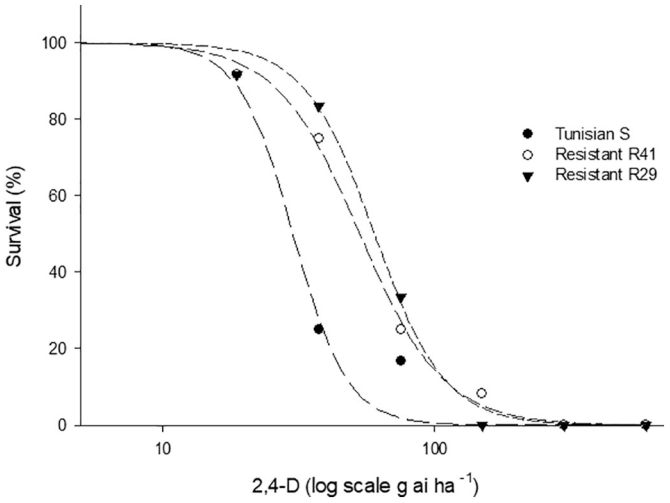


**Fig. 3.** Dose-response curves represented in percentages of survival for dicamba and dicamba plus Malathion (M) in *Sinapis alba* populations, R41, R29 and susceptible (S).

**Table 6**
Frequency (%) of ALS alleles identified at positions 197 and 574 in three different *S. alba* populations, R41, R29 and susceptible (S). Numbers in parentheses: plants sequenced per population.

| Mutation position | AA | | R41 (20) | R29 (20) | S (10) |
|---|---|---|---|---|---|
| 574 | W/L | Heterozygote | 35 | 35 | – |
| | | Homozygote | 10 | 30 | – |
| 197 | P/S | Heterozygote | 25 | 10 | – |
| | | Homozygote | 5 | 5 | – |
| 197 + 574 | W/L + P/S | Heterozygote | 5 | 10 | – |
| No mutation | | | 20 | 10 | 100 |

**Table 5**
Estimated parameters (means ± standard errors) for survival for dicamba and dicamba plus malathion (M) in *Sinapis alba* populations, R41, R29 and susceptible (S).

| HERBICIDE | POPULATION | SLOPE | LC50 | F | $R^2$ | P |
|---|---|---|---|---|---|---|
| | Tunisian S | −2.18 ± 0.71 | 41.03 ± 7.791 | 1 | 0.989 | <0.0001 |
| | R41 | −2.17 ± 0.36 | 103.12 ± 4.43 * | 2.513 | 0.987 | <0.0001 |
| dicamba | R29 | −4.56 ± 2.58 | 75.63 ± 7.93 * | 1.843 | 0.993 | <0.0001 |
| | Tunisian S | −2.18 ± 0.74 | 41.03 ± 7.53 ns | 1 | 0.978 | <0.0001 |
| dicamba + (M) | R41 | −1.62 ± 3.19 | 36.66 ± 3.467 † | 0.872 | 0.960 | 0.0002 |
| | R29 | −1.19 ± 0.58 | 49.57 ± 6.233 † | 1.17 | 0.919 | <0.0001 |

LC50 = effective dose required for 50% reduction in survival, RF = resistance Factor (LC50 R/LC50 S), $R^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares), *P* value = probability level of significance of the non-linear model. ns: non-significant difference; *: significant difference compared to Tunisian Susceptible ($p < 0.05$); †: significant difference compared to Dicamba without M ($p < 0.05$).

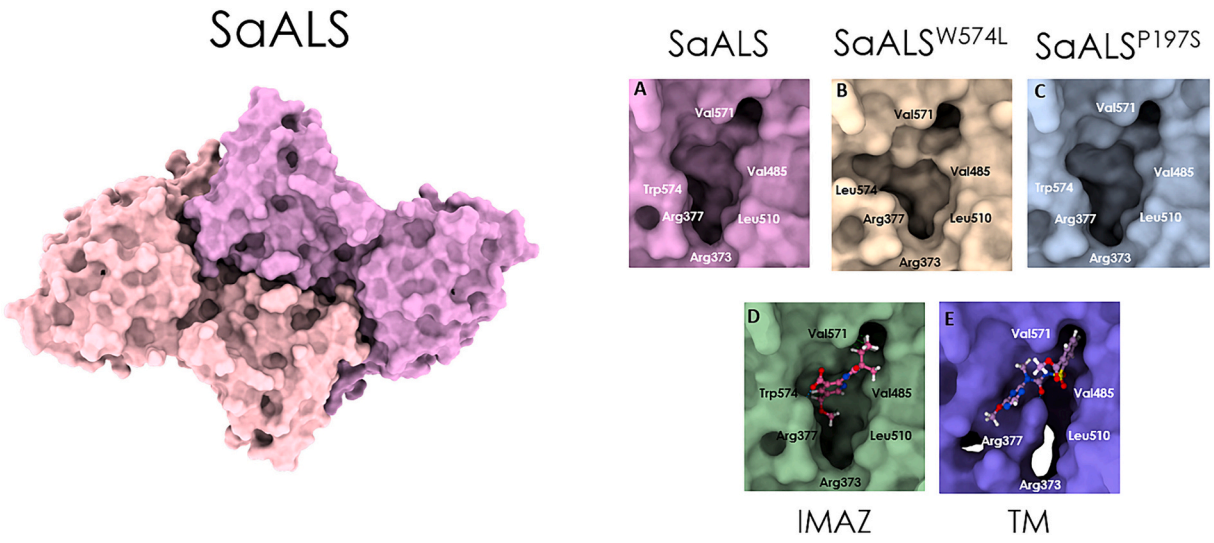


**Fig. 4.** Comparison of the herbicide binding pocket of predicted structures for *Sinapis alba* ALS enzyme (SaALS): SaALS model (dimeric) (left), Surface of the herbicide binding site of SaALS (A), SaALS$^{W574L}$ (B) and SaALS$^{P197S}$ (C) and herbicide bound to SaALS docking prediction imazamox (IMZ) (E) and tribenuton-methyl (TM) (F) into the SaALS structural models (right).

investigations in *S. alba* from Spain have unveiled the genetic basis for resistance to TM, manifesting as a singular amino acid substitution of proline to serine at position 197, attributed to a mutation from codon CCT to TCT in domain A of the ALS gene (Cruz-Hipolito et al., 2013). Additionally, recent research has disclosed an Asp376Glu substitution in the *ALS* gene of Spanish *S. alba* (Palma-Bautista et al., 2022b), which confers a wide-ranging resistance profile against various ALS inhibitors (Tranel et al., 2022b). One of the particular interests in this study is the identification of the Trp574Leu mutation, which represents the first documentation of this specific mutation in *S. alba*. It's one of the most well-studied mutations, which is well-located inside the herbicide binding site, disrupting a key hydrogen bond network that is important for herbicide binding (Lonhienne et al., 2022). The presence of mutations in two different positions in the *ALS* gene in these *S. alba* populations also indicate the remarkable genetic diversity and adaptability of this species. In addition, in 4 of the individuals included in this study it has been found that had the two mutations (Pro197-Ser plus Trp574-Leu), both mutated sites showing a double signal peak. This has been previously reported in *Capsella bursa-pastoris* (Lu et al., 2023), conferring higher resistance in those "double-mutated" R plants.

This study is the first to attempt a molecular docking analysis with the SaALS enzyme, and the individual effects of two amino acid substitutions in two positions on the binding interactions with TM and imazamox. The simulation of the ligand receptor interactions revealed that the Pro197Ser and Trp574Leu mutations have distinct impacts on the interactions between SaALS and herbicides. Regarding Pro197Ser mutation, the TM aromatic ring cannot properly bind due to a reduction in the number of hydrogen bonds. However, docking analysis showed that imazamox can still bind to SaALS with the Pro197Ser mutation, unlike other ALS inhibiting chemistries, which makes this mutation usually selective to SU, as reported in similar studies (Palma-Bautista et al., 2022b). However, the Trp574Leu mutation has been associated with broad ALS inhibiting herbicide resistance in various weed species (Tranel et al., 2022b). In line with this, the sequencing results showed that plants surviving the four ALS inhibitors families tested could harbor the Trp574Leu mutation. This finding supports the hypothesis that the new mutation in *S. alba* could provide cross-resistance to IMI and SU herbicides, as predicted by our docking analysis. This validation of our predictions is an important step towards understanding the TSR mechanisms of herbicide resistance to ALS inhibitors in this species.

Moreover, it is interesting to note that a substantial proportion of the examined plant specimens exhibit heterozygosity for the two mutations detected. *S. alba* is an obligate outcrossing species and this can lead to the heterozygosity observed. The presence of some plants harboring both mutations also highlights this allogamous nature of the species. It is very unlikely that hybridization events with Clearfield® rapeseed, a crop subjected to biennial cultivation cycles, alternating between imazamox application in the rapeseed year and the use of TM or SU herbicides in the cereal year, may contribute to these observations. The risk for gene flow between genetically modified oil rapeseed and close relatives has been demonstrated very low (Song et al., 2010), as demonstrated for other Clearfield® crops (Engku et al., 2016). Moreover, a compilation of cross ability between species in the Brassicaeae was published, showing there is almost no crossing between *S. alba* and *B. napus* (FitzJohn et al., 2007). Finally, the occurrence of natural hybridization between distant relatives in natural conditions is low (Katche et al., 2019).

This investigation highlights the presence of remarkable genetic and mechanistic diversity within both individual plants and populations. It is evident that certain individuals exhibited resistance to ALS herbicides without discernible genetic mutations. In this study, the reduction of resistance to TM and imazamox in populations R41 and R29 was only significant at particular rates. These results indicate that P450 may be involved in the resistance, but are not conclusive. The increased activity of P450s can lead to accelerated herbicide metabolism, reducing the effective concentration of herbicide action within plant tissues (Jugulam and Shyam, 2019). Previous studies, particularly in *S. alba* have shown that P450s can be part of herbicide breakdown (Palma-Bautista et al., 2022a), which aligns with our own research findings. Moreover, metabolism studies, showed that the TM is rapidly transformed into harmless substances in resistant plants and that both P450 and glucosyltransferases (GS) enzymes play crucial roles in this process (Cruz-Hipolito et al., 2013; Palma-Bautista et al., 2022b). It is noteworthy that metabolic processes, whether acting independently or in concert with genetic mutations, significantly contribute to the overarching phenomenon of broad resistance to ALS inhibitors, further enhancing the complexity of herbicide resistance dynamics. For instance, a study conducted in individual plants of *P. rhoeas* exhibited a dual resistance mechanism, including both TSR and NTSR, with a notable emphasis on the latest, which predominantly involved enhanced metabolic processes (Rey-Caballero et al., 2017).

On the other hand, our investigation provides substantial evidence for the risk of evolving resistance of both populations to auxin herbicides, encompassing 2,4-D and dicamba. Despite resistance factors were

not higher than 4 as defined by HRAC (2024) for both auxins, this resistance is more pronounced in one of the populations, particularly evident in the case of R41 resistant to dicamba. Furthermore, our findings reveal a subtle yet significant synergistic interaction between malathion and dicamba. This result is not in line with the results of other researchers, who found that the herbicides 2,4-D and MCPA could effectively manage ALS resistant populations of *S. alba* (Palma-Bautista et al., 2022a). This observation strongly suggests the involvement of enhanced metabolic processes mediated by P450s in the evolution of cross-resistance to auxin herbicides. It is plausible that a single P450 enzyme, similar to observations in other broad-leaf species like *P. rhoeas* (Torra et al., 2021), might be responsible for conferring cross-resistance to both ALS and auxin herbicides. This intriguing possibility underscores the complex interplay of resistance mechanisms and warrants further In-depth investigation within the context of scientific research.

In brief, this research is confirming the third herbicide resistance case in a Tunisian weed species. Both TSR and NTSR mechanisms confer broad resistance to all ALS inhibitors in *S. alba*. For TSR, among two different altered positions found, the Trp574Leu is reported for the first time in this species, while for NTSR, evidences support the involvement of P450s in auxins and ALS-inhibiting herbicides resistance. Moreover, this study supports that the current situation in Tunisia presents a high risk of selecting multiple herbicide-resistant *S. alba* populations due to the widespread use of ALS and auxin mimic herbicides, coupled with the emergence of ALS inhibitor-resistant populations in cereals and rapeseed. This highlights the urgent need for effective management strategies to either prevent or delay the spread of herbicide resistance in weed populations. This emphasizes the pressing requirement for efficient management strategies, particularly in regions where wheat and rapeseed (especially Clearfield® varieties) are prevalent and ALS and auxin mimic herbicides are extensively used.

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## CRediT authorship contribution statement

**Myriem Chtourou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria D. Osuna:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Conceptualization. **José G. Vázquez-García:** Writing – review & editing, Visualization, Validation. **Jorge Lozano-Juste:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Investigation. **Rafael De Prado:** Writing – review & editing, Visualization, Validation, Supervision. **Joel Torra:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Thouraya Souissi:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2024.105882.

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