

Article

Occurrence and Mechanisms Conferring Multiple Resistance to ALS-Inhibiting and Auxins Mimics Herbicides in *Papaver rhoeas* from Tunisia

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Abstract: Herbicide-resistant corn poppy (*Papaver rhoeas* L.) is one of the most important broadleaved weeds and the number of resistant cases is still growing. The aims of this study were to confirm the resistance of *P. rhoeas* from Tunisia to ALS inhibitors and auxin mimics and investigate the mechanisms of Target-Site Resistance (TSR) and Non-Target Site Resistance (NTSR) involved. Dose-response trials to determine cross-resistance patterns for ALS inhibitors and auxin mimics were conducted in a greenhouse. In this study, multiple resistance to tribenuron-methyl and dicamba but not to 2,4-D was found in *P. rhoeas* populations. Cross-resistance to imazamox was confirmed as well. Sequence analysis of the *ALS* gene detected target-site mutations in codon 197 of the *ALS* gene, namely, Pro197His, Pro197Thr, Pro197Leu, and Pro197Asn. In this study, the metabolism experiments with malathion (a cytochrome P450 inhibitor) showed that malathion reduced resistance to imazamox, indicating that P450 is involved in the resistance. TSR and NTSR mechanisms to ALS inhibitors likely coexist. The findings of this study revealed a significant synergistic interaction between malathion and dicamba in particular populations, suggesting that the resistance to auxin mimics can be conferred by enhanced metabolism.

Keywords: synthetic auxin herbicides; ALS inhibitor; enhanced metabolism; target site mutation



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1. Introduction

Weeds cause significant crop losses by competing for nutrients, water, or light [1]. Several factors, including soil cultivation, crop rotation, fertilization, and irrigation, can influence the populations of weeds [1]. The use of herbicides is currently considered a critical management approach in agronomic crops. At present, there are approximately 150 chemical compounds deployed to combat weeds, encompassing 25 distinct action sites [2]. However, herbicide use is associated with the rapid evolution of herbicide resistance. This phenomenon is based on weeds' ability to survive and keep growing despite the application of a dose of herbicide that is considered lethal [3], and is mainly caused by simplified crop rotations and excessive use of the same active substances or herbicides with the exact mechanism of action. As a result of selection pressure, the number of resistant weeds is growing, which in turn leads to higher crop production costs [4]. The problem of weed resistance to herbicides is becoming increasingly common. Worldwide, resistant biotypes have been identified and herbicide resistance has now emerged in 272 plant species globally [5]. In winter crops in Tunisia, there are suspected resistance concerns for corn poppy.

Corn poppy (*Papaver rhoeas* L.) is a prevalent winter crop weed across Europe [6]. It belongs to the Papaveraceae family and is particularly dominant in winter wheat fields, causing significant yield losses. In addition to winter wheat, it poses a serious threat to barley and winter oilseed rape in some regions [7]. In temperate climates, *P. rhoeas* can emerge in autumn and spring, and their prolonged emergence makes it more difficult to control [8]. Herbicide-resistant *P. rhoeas* is identified as one of Europe's most significant dicotyledonous weed concerns by various researchers [9,10]. The rise in monoculture farming of cereals, coupled with extensive use of 2,4-Dichlorophenoxyacetic acid (2,4-D) since the 1960s, followed by the introduction of tribenuron-methyl in the early 1980s, has led to the selection of acetolactate synthase (ALS) inhibitors and/or 2,4-D resistant biotypes of *P. rhoeas* [10,11]. So far, *P. rhoeas* biotypes resistant to auxin mimics or ALS inhibitors have been identified in ten European countries [5], and the number of new cases of herbicide-resistant biotypes for this species is still growing [12]. Resistance to these herbicides can be due to target-site resistance (TSR) or non-target-site resistance (NTSR) mechanisms. TSR mechanisms largely involve mutation(s) in the herbicide's target site of action, while NTSR mechanisms usually result from enhanced metabolism of the herbicide [13,14]. The situation is complex in *P. rhoeas*-resistant biotypes to ALS inhibitors. TSR only [15,16], NTSR only [17] or both mechanisms of resistance can be observed and, in some cases, even in the same plant [18].

In populations from Greece, France, Italy, Spain, and the UK, an ALS gene mutation in codon Pro197 (resulting in substitutions of Leu, His, Arg, Ser, and Thr) was observed to confer resistance to tribenuron-methyl [15,16,18,19]. However, a recent study reported *P. rhoeas* amino acid substitution Pro197 by Phe due to a double-nucleotide change. This double mutation could confer cross-resistance to most ALS inhibitors [20]. Mutations in codon 574 have been found much less frequently, but mutations in codons 197 and 574 have been reported in one French *P. rhoeas* population [21]. TSR is a commonly observed resistance mechanism in *P. rhoeas* [22]; however, resistance to ALS inhibitors in this species may be also due to an NTSR mechanism, which usually results from an enhanced metabolism of the herbicide [17]. Studies examining the metabolism of imazamox have confirmed the presence of enhanced metabolism in certain Spanish populations [14]. Both TSR and NTSR mechanisms to ALS-inhibiting herbicides can coexist in the same population or even in an individual *P. rhoeas* plant [17]. NTSR is the only proposed mechanism of *P. rhoeas* resistance to 2,4-D [17]. To date, reduced herbicide uptake, reduced translocation, and detoxification are recognized as being the main NTSR mechanisms of resistance to 2,4-D in weeds [23]. The detection of two hydroxy metabolites in the shoots and roots of resistant *P. rhoeas* biotypes in a previous study [24] confirms that the enhanced metabolism of 2,4-D is the mechanism of resistance. This and subsequent studies indicated that enhanced metabolism was mediated by the Cytochrome P450 (Cyt. P450) enzyme family [14].

The presence of resistant poppies in fields significantly threatens the quality [24]. This study was conducted in response to the failure of chemical control of *P. rhoeas* by farmers in northeast Tunisia. It aims to investigate the occurrence of resistance to ALS inhibitors and auxin mimics and determine the mechanism(s) involved in the resistance of *P. rhoeas* populations harvested from wheat fields.

2. Materials and Methods

2.1. Plant Material

Twelve populations of *P. rhoeas* were collected in the summer of 2020 from wheat fields in Northern Tunisia that have been repeatedly treated with ALS-inhibiting herbicides and auxin mimics. 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. Seeds were collected, scarified manually, and stored at 4 °C until experiments. All populations had undergone a preliminary screening with the herbicides described in Section 2.2 at the field rate. Among the 12 populations, 6 suspected resistant (R) populations (R1, R2, R3, R4, R5, and R6) of *P. rhoeas* were selected for all of the experiments.

2.2. Chemicals

The herbicides used for the whole-plant greenhouse trials were those listed in Table 1. The application process involved using a precision bench sprayer equipped with flat fan 110° nozzle tips (Low drift, ISO LD-02-110-CT Yellow syntal, HARDI®) positioned at a height of 50 cm and operating at a pressure of 250 kPa. The sprayer was calibrated to deliver a volume of 200 L per hectare.

Table 1. Main characteristics of the herbicides used in this study.

Site of Action	Herbicide	Company	Commercial Product	Concentration	Formulation
ALS inhibitor	florasulam	Corteva	Nikos®	5% w/v	SC
	imazamox	BASF	Pulsar 40®	4% w/v	SL
	tribenuron-methyl	DuPont	Granstar SX®	50% w/w	SG
Auxin mimics	2,4-D	Dow AgroSciences	Esteron 60®	60% w/v	SL
	dicamba	Syngenta	Banvel® D	48% w/v	SL

2.3. Cross and Multiple-Resistance to Herbicides

The germination of mature *P. rhoeas* seeds was conducted in aluminum trays containing only peat and distilled water. The trays were subsequently placed in a growth chamber programmed to maintain a temperature cycle of 28/18 °C (day/night), with a 16-h photoperiod, 80% relative humidity, and a photosynthetic photon flux of 850 µmol m⁻² s⁻¹. Once the seedlings reached the 4-leaf stage, they were transferred into plastic pots (7 cm × 7cm × 9 cm) containing a sand and peat moss mixture in a volumetric ratio of 1:1, and each pot contained two plants. The pots were then transferred to a greenhouse located at the University of Lleida (Spain). Various doses of all the herbicides described in Section 2.2 were applied, as detailed in Table 2. Plants were monitored until 28 days after treatment (DAT). The weight of the survivors was determined by harvesting the plants at ground level. Fresh weight data were expressed as percentages relative to untreated control plants. The experiment was replicated two times, with each combination of treatment and population having five replicates.

Table 2. Herbicide range of doses applied to *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S).

Herbicide	Field Rate g ai ha ⁻¹	Pop	Rates g ai ha ⁻¹									
florasulam	7.5	R1, R2, R3, R4, R5, R6 S	0.46	0.93	1.875	3.75	7.5	15	30			
			0.46	0.93	1.875	3.75	7.5	15	30			
imazamox	40	R1, R2, R3, R4, R5, R6 S	2.5	5	10	20	40	80	160			
			0.62	1.25	2.5	5	10	20	40			
tribenuron-methyl	18.7	R1, R2, R3, R4,	2.33	4.67	9.35	18.7	37.4	74.8	149.6	299.2	598.4	1196.8
		R5, R6	18.7	37.4	74.8	149.6	299.2	598.4	1196.8			
		S	0.29	0.58	1.16	2.33	4.67	9.35	18.7			
2,4-D	600	R1, R2, R3, R4, R5, R6 S	37.5	75	150	300	600	1200	2400			
			18.75	37.5	75	150	300	600				
dicamba	144	R1, R2, R3, R4, R5, R6 S	9	18	36	72	144	288	576			
			4.5	9	18	36	72	144				

2.4. Malathion Effect

A screening trial was conducted utilizing a Cyt. P450 inhibitor, malathion, on selected *P. rhoeas* populations showing the highest resistance factors. Plants from the S, R3, and R4 populations were subjected to malathion treatment at a rate of 1000 g ai ha⁻¹ and

left at room temperature for one hour. Then, whole-plant dose–response experiments were conducted with imazamox, as described in Section 2.3. For dicamba and 2,4-D, only populations R4 and R6 were selected. Two control groups were included: one comprising non-treated plants and the other consisting of plants treated solely with malathion. After twenty-eight days, fresh weight measurements were taken and analyzed to determine the reduction compared to the untreated control.

2.5. ALS Gene Sequencing

2.5.1. DNA Isolation

During this step, four plants were selected for each of the six R population surviving field rates of TM, with eight for the S one. The plant samples were preserved by immersion in liquid nitrogen (N₂), followed by lyophilization using a LyoQuest apparatus (Model H140, BeiJie, Telstar, Terrassa, Barcelona) at a temperature of -50°C and pressure ranging between 10 and 0.03 mBar for a duration of 48 h. Subsequently, approximately 20 mg of lyophilized tissue per sample was employed for DNA extraction, utilizing the Speedtools Plant DNA Extraction Kit (manufactured by Biotools B&M Labs S.A., situated in Valle de Tobalina, Madrid, Spain). Each sample's DNA concentration was quantified using a NANODROP Thermoscientific spectrophotometer (manufactured by ThermoFisher, Nano-Drop Products, Wilmington, DE, USA).

2.5.2. PCR Amplification

The conserved domains were amplified using two different sets of primers. The forward primer was used to target the domains C, A, and DUP02 (5'-GAAACACCCATTTCCA CCAC-3') and the reverse primer Down02 (5'-TGGGTGCTCGAGACATATACC-3') as described [25]. Conversely, domains B and E were amplified employing the forward primer (PaF) (5'-CTGCCGTTGCTAAACCTGA-3') and the reverse primer (PaR) (5'-AAGACCTAG CAAGCTGAGAGAC-3'). The PCR conditions were consistent for both primer sets, except for the annealing temperature.

2.5.3. Gene Sequencing

Following PCR amplification, the resulting products were observed under ultraviolet light (wavelength: 320 nm) utilizing the ALPHA DIGI DOC Pro instrument manufactured by Alpha Innotech Corporation, based in Johannesburg, South Africa. Subsequently, the bands corresponding to the amplicons were excised and purified employing the Speed Tools PCR Clean-Up Kit (produced by Biotools, B&M Labs, located in Madrid, Spain). The purified samples were then forwarded to an external laboratory, STABVIDA, situated in Caparica, Portugal, for sequencing. The obtained sequencing data were analyzed and visualized utilizing CHROMAS software 2.6.6.0. Further analysis involved aligning these sequences using CLUSTAL OMEGA software 11.0.13.

2.6. Statistical Analysis

There were five duplicates of each treatment in the fully randomized design of the studies. There were two plants in a single pot that served as the experimental unit. Using Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA), dose–response data from the entire plant studies were analyzed. The data were fitted to a nonlinear log logistic regression model [26] in the manner described below:

$$Y = a / (1 + \exp(-(x - g)/b)) \quad (1)$$

where b is the response line slope, g is the herbicide dose at the point of inflection halfway between both the upper and lower asymptotes (representing the ED₅₀), and x (the independent variable) is the herbicide dose. Y is the fresh aboveground weight expressed as a percentage of the untreated control. a is the coefficient corresponding to the upper asymptotes (100). Using the formula $\text{RF}_{50} = \text{ED}_{50} \text{ of R} / \text{ED}_{50} \text{ of S}$, the resistance factor (RF) was determined as the ratio of the ED₅₀ values for the resistant (R) biotypes to the

susceptible (S) biotype. ED_{90} (the dose at which 90% of efficacy can be observed) was determined and the resistance factor (RF_{90}) was calculated at ED_{90} using the following ratio: $ED_{90}R/ED_{90}S$. The ED_{50} values between S and R populations for the Whole-Plant Dose–Response Assays were submitted to an analysis of variance (ANOVA), and Tukey’s test was conducted to compare the means for all herbicides. Additionally, the ED_{50} values between malathion treatments for each population in the auxins and imazamox metabolism assays were also compared using ANOVA. Significant differences were defined as those with $p < 0.05$. Normality was checked using the Shapiro–Wilk test and the homogeneity of variance condition was also confirmed with the Spearman rank correlation between the absolute values of the residuals and the observed value of the dependent variable.

3. Results

3.1. ALS-Inhibiting Herbicides

3.1.1. Whole-Plant Dose–Response Assays

The results of the whole-plant dose–response bioassays to ALS inhibitors indicated that all the (R) populations showed resistance, or at least reduced susceptibility, to the active ingredients tested compared to the (S) population. The six *P. rhoeas* populations tested during this study were found to be resistant to tribenuron-methyl based on the percentages of fresh weight reduction in treated plants. The results showed ED_{50} values ranging from 34.78 g ai ha^{−1} to 118.54 g ai ha^{−1} and RF_{50} varying from 77.28 to 263.42 (Figure 1, Table 3). Resistance to tribenuron-methyl was observed in all populations considering the ED_{90} , which showed a high resistance level even at very high herbicide doses. The RF_{90} was at least 9.24.

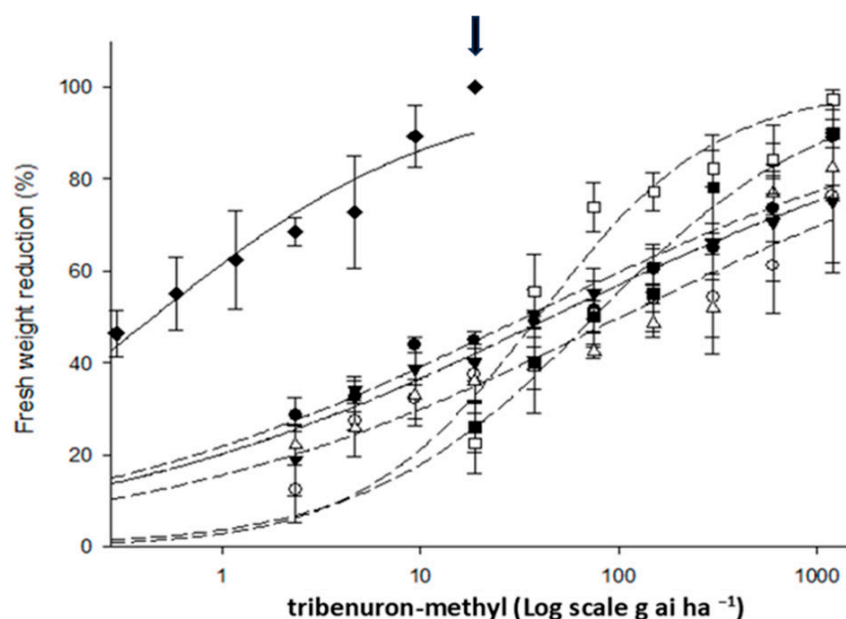


Figure 1. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for tribenuron-methyl in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S). Solid line denotes the S population, while short-dashed lines denote the R populations. The arrow denotes the recommended field rate of tribenuron-methyl.

Regarding resistance to florasulam, no significant differences were found in all putative populations compared to the S population (Figure 2, Table 4). The double field rate dose of florasulam caused fresh weight reductions of at least 90% in the six (R) populations (R1, R2, R3, R4, R5, and R6) with RF_{50} around 1 and ED_{50} ranging from 0.37 to 0.62 g ai ha^{−1} (Figure 2, Table 4). However, only population R4 showed a different pattern and responded differently to the application of this active ingredient with RF_{90} 4.24.

Table 3. Estimated parameters for non-linear regressions for fresh weight reduction for tribenuron-methyl in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S).

Herbicide	Population	Slope	ED ₅₀	RF ₅₀	ED ₉₀	RF ₉₀
tribenuron-methyl	S	2.56 ± 0.09	0.45 ± 0.10	1	18.2998	1
	R1	0.44 ± 0.05	34.78 ± 8.84 *	77.28	5129.35	225.34
	R2	0.59 ± 0.13	118.54 ± 3.99 *	263.42	11,188.74	491.55
	R3	0.44 ± 0.05	43.909 ± 6.99 *	97.55	6475.70	284.49
	R4	0.47 ± 0.07	81.00 ± 8.80 *	180	8685.36	474.63
	R5	0.45 ± 0.06	84.77 ± 8.18 *	188.37	501.84	14.4
	R6	1.35 ± 0.39	45.80 ± 14.65 *	101.71	168.99	9.24

ED₅₀ and ED₉₀ represent the effective doses required for 50% and 90% reductions in plant biomass, respectively. RF₅₀ = resistance factor (ED₅₀ R/ED₅₀ S). RF₉₀ = resistance factor (ED₉₀ R/ED₉₀ S). The R² values range from 0.94 to 0.99 and the *p* values (probability level of significance of the non-linear model) are <0.0001. * = significant differences between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

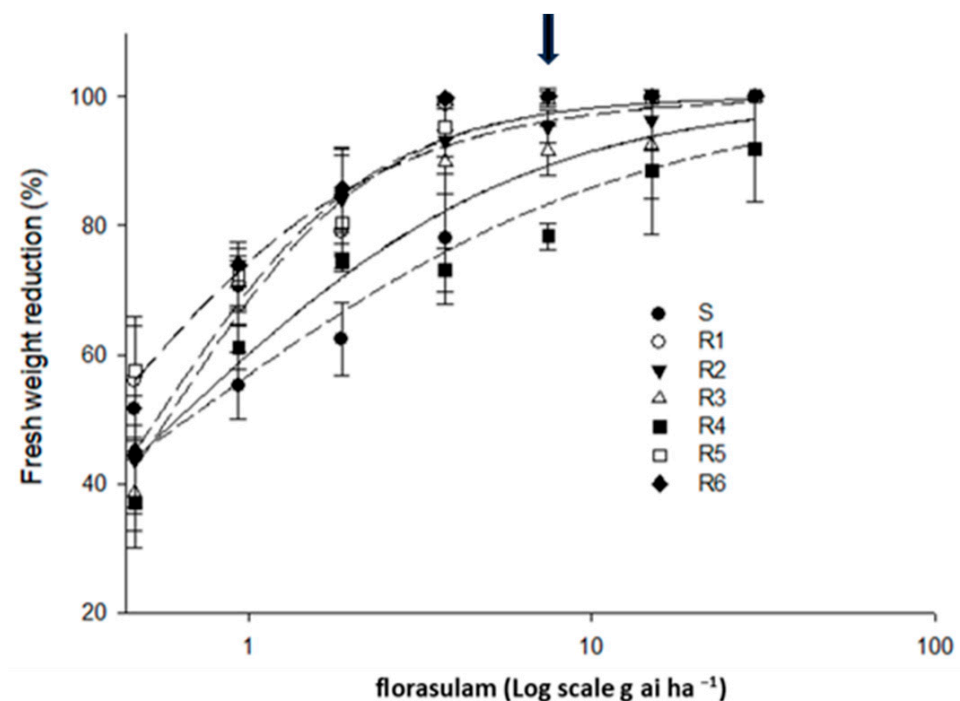


Figure 2. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for florasulam in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S). Solid line denotes the S population and short-dashed lines denote the R populations. The arrow denotes the recommended field rate of florasulam.

Table 4. Estimated parameters for non-linear regressions for fresh weight reduction for florasulam in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S).

Herbicide	Population	Slope	ED ₅₀	RF ₅₀	ED ₉₀	RF ₉₀
florasulam	S	2.5 ± 0.07	0.40 ± 0.17	1	5.67	1.00
	R1	1.47 ± 0.24	0.41 ± 0.06 ns	1.02	2.59	0.9
	R2	1.37 ± 0.09	0.54 ± 0.02 ns	1.35	2.49	0.43
	R3	1.89 ± 0.86	0.58 ± 0.06 ns	1.45	4.16	0.73
	R4	2.84 ± 0.74	0.62 ± 0.15 ns	1.54	24.09	4.24
	R5	1.07 ± 0.13	0.37 ± 0.04 ns	0.92	3.12	0.55
	R6	1.67 ± 0.14	0.52 ± 0.01 ns	1.3	5.76	1.01

ED₅₀ and ED₉₀ represent the effective doses required for 50% and 90% reductions in plant biomass, respectively. RF₅₀ = resistance factor (ED₅₀ R/ED₅₀ S). RF₉₀ = resistance factor (ED₉₀ R/ED₉₀ S). The R² values range from 0.93 to 0.99, and the *p* values (probability level of significance of the non-linear model) are <0.0001. ns = non-significant difference between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

The ED_{50} values of the susceptible population of *P. rhoeas* to imazamox was $0.78 \text{ g ai ha}^{-1}$. Among the six R populations, no significant difference was found in R5 compared to the susceptible population. A low resistance was observed in three populations: R1, R2, and R6, with resistance factors (RF_{50}) ranging from 2.82 to 6.17 (Figure 3, Table 5). Furthermore, the ED_{50} values were less than half of the recommended field rate for this active ingredient. However, the RF_{50} for populations R3 and R4 was approximately 11 for both populations, and the ED_{50} value was $8.73 \text{ g ai ha}^{-1}$ for R3 and slightly higher for R4 at $9.01 \text{ g ai ha}^{-1}$. With regard to ED_{90} , populations R3 and R4 proved to be more resistant (with RF_{90} around 20) than R1, R2, R5, and R6, which showed lower RF_{90} ranging from 3.09 to 8.54.

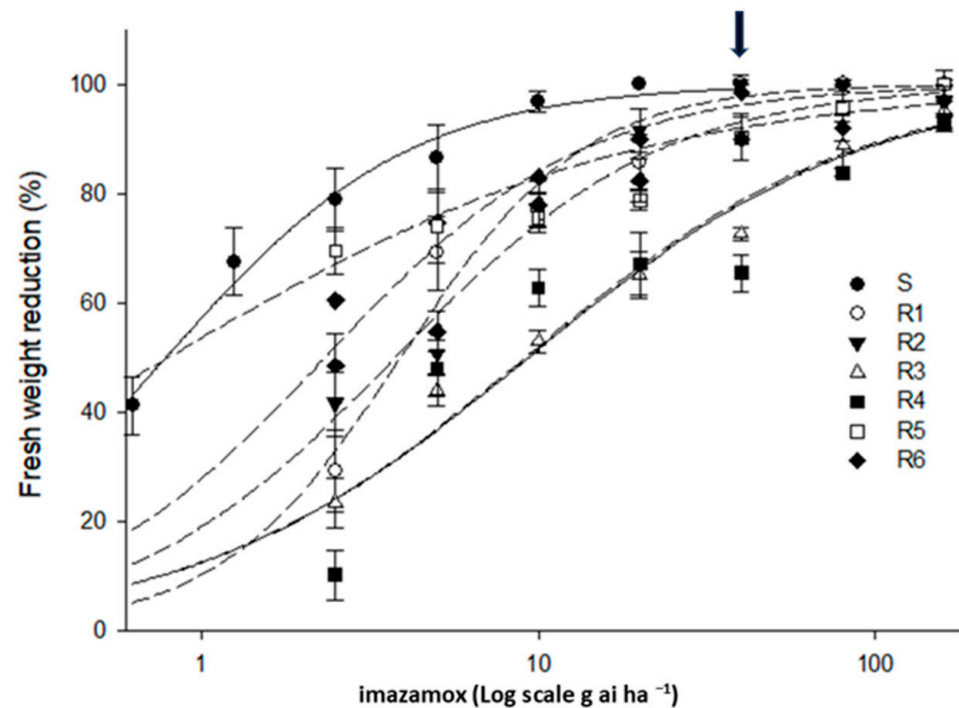


Figure 3. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for imazamox in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S). Solid line denotes the S population and short-dashed lines denote the R populations. The arrow denotes the recommended field rate of imazamox.

Table 5. Estimated parameters for non-linear regressions for fresh weight reduction for imazamox in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S)

Herbicide	Population	Slope	ED_{50}	RF_{50}	ED_{90}	RF_{90}
imazamox	S	1.1 ± 0.09	0.78 ± 0.04	1	4.98	1.00
	R1	1.49 ± 0.21	$3.83 \pm 0.41 *$	4.91	16.89	3.39
	R2	1.29 ± 0.13	$3.92 \pm 0.39 *$	5.02	15.42	3.09
	R3	0.81 ± 0.07	$8.73 \pm 0.82 *$	11.19	115.61	23.21
	R4	0.91 ± 0.20	$9.01 \pm 2.46 *$	11.55	109.52	21.99
	R5	0.51 ± 0.01	$2.2 \pm 0.80 \text{ ns}$	2.82	27.38	5.59
	R6	0.56 ± 0.03	$4.82 \pm 1.16 *$	6.17	42.57	8.54

ED_{50} and ED_{90} represent the effective doses required for 50% and 90% reductions in plant biomass, respectively. RF_{50} = resistance factor ($ED_{50} \text{ R} / ED_{50} \text{ S}$). RF_{90} = resistance factor ($ED_{90} \text{ R} / ED_{90} \text{ S}$). The R^2 values range from 0.83 to 0.98, and the p values (probability level of significance of the non-linear model) are <0.0001 . ns = non-significant difference and * = significant differences between S and resistant populations by ANOVA ($p < 0.05$). $\pm$ denotes standard errors.

3.1.2. Sequencing

No amino-acid replacements at positions 197 and 574 were found in the S plants (Table 6). However, although four plants were sequenced per population, a variety of

mutations were already found, and four amino-acid substitutions were identified at amino acid Pro197. Pro197His was found in R1 and R2 populations, Pro197Asn in populations R5 and R6, and Pro197Leu in R4 and R6 populations. Pro197Thr was found in all R populations. Also, overlapping peaks were observed in the chromatograms showing that some of the R plants were heterozygous for those mutations.

Table 6. Presence of different amino acid changes at positions 197 and 574 in different *Papaver rhoeas* populations.

Position		197						574
AA ^a	Pro/Pro	Pro/Arg	Pro/His	Pro/Leu	Pro/Ser	Pro/Asn	Pro/Thr	Pro/Leu
S	+	-	-	-	-	-	-	-
R1	-	-	+	-	-	-	+	-
R2	-	-	+	-	-	-	+	-
R3	-	-	-	-	-	-	+	-
R4	-	-	-	+	-	-	+	-
R5	-	-	-	-	-	+	+	-
R6	-	-	-	+	-	+	+	-

^a AA = amino acid, - No mutation found at this position, + Mutation found at this position, (*) one individual plant shows heterozygosity for both mutations.

3.1.3. Malathion Effect

As shown in Figure 4, both populations R3 and R4 of *P. rhoeas* showed a significant difference between plants treated with imazamox and those treated with a combination of imazamox and malathion. The ED₅₀ decreased to 4.32 g ai ha⁻¹ for R3 and 4.96 g ai ha⁻¹ for R4, from initial values of 9.71 g ai ha⁻¹ and 11.85 g ai ha⁻¹, respectively. Furthermore, the resistance factor for both populations decreased by at least two-fold when compared to plants treated solely with imazamox (Figure 4, Table 7).

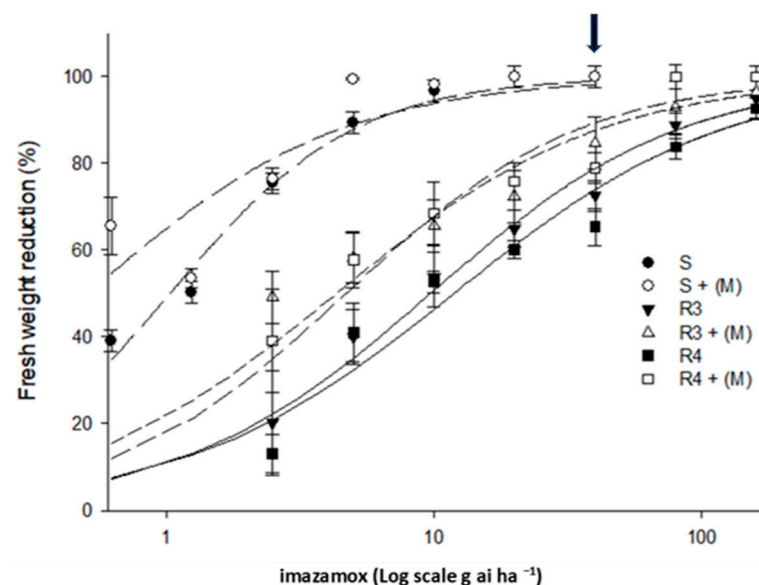


Figure 4. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for imazamox and imazamox plus malathion (M) at 1000 g ai ha⁻¹ in *Papaver rhoeas* populations, R3, R4, and susceptible (S). Long-dashed lines denote S population for imazamox and imazamox +M, solid line denotes the R populations for imazamox, and short-dashed lines denote the R populations for imazamox +M. The arrow denotes the recommended field rate of imazamox.

Table 7. Estimated parameters for fresh weight reduction for imazamox and imazamox plus malathion (1000 g ai ha^{−1}) in *Papaver rhoeas* populations R3, R4, and susceptible (S).

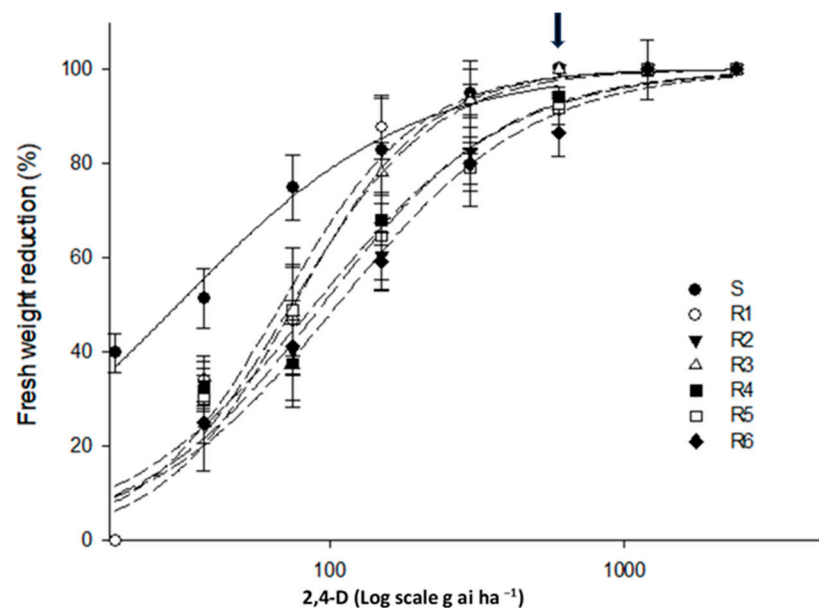
Herbicide	Population	Slope	ED ₅₀	RF ₅₀
imazamox	S	1.20 ± 0.11	1.03 ± 0.06	1
	+Malathion	0.95 ± 0.31	0.99 ± 0.21	1
	R3	0.92 ± 0.09	9.71 ± 1.04	9.42
	+Malathion	0.87 ± 0.16	4.32 ± 0.92 *	4.19
	R4	0.89 ± 0.12	11.85 ± 1.94	11.50
	+Malathion	0.95 ± 0.17	4.69 ± 0.83 *	4.73

ED₅₀ = effective dose required for 50% reduction in plant biomass, RF = resistance factor (ED₅₀ R/ED₅₀ S). The R² values range from 0.92 to 0.98, and the *p* values (probability level of significance of the non-linear model) are <0.0001. * = significant differences between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

3.2. Auxins

3.2.1. Dose–Response Experiment

After the initial days of 2,4-D treatment, all six (R) *P. rhoeas* populations showed similar behavior: epinasty appeared, growth was reduced, and morphological damage was observed at the recommended rate (600 g ai ha^{−1}). They showed a significant response to 2,4-D when compared to the S population (Figure 5, Table 8). The resistance levels according to 50% of fresh weight reduction were very low for R1, R3, and R5 at around 2. Slightly higher RF₅₀ values were observed for R2, R4, and R6, around 3. Considering the RF₉₀ too, no resistance to 2,4-D was considered in all populations.

**Figure 5.** Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for 2,4-D in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S). Solid line denotes the S population and short-dashed lines denote the R populations. The arrow denotes the recommended field rate of 2,4-D.

In relation to the dicamba herbicide, notable differences were observed among the R biotypes of *P. rhoeas*. The suspected resistant populations, R1 and R2, presented overlapping characteristics with almost negligible resistance factors (<1) and ED₅₀ values of 21.63 g ai ha^{−1} and 22.57 g ai ha^{−1}, respectively (Figure 6, Table 9). These values are closer to the dicamba application rate required to achieve a 50% reduction in fresh weight for the S population, which was 22.76 g ai ha^{−1}. In contrast, R3, R5, and R6 demonstrated low resistance levels considering 50% of biomass reduction, with RF₅₀ of 1.72, 1.68, and

2, respectively, and ED₅₀ values ranging from 38.34 g ai ha⁻¹ to 45.65 g ai ha⁻¹. Notably, only population R4 showed a substantially higher RF₅₀ of 3.56 and a higher ED₅₀ of 81.23 g ai ha⁻¹. The RF₉₀, considering a 90% reduction in fresh weight, confirmed these results.

Table 8. Estimated parameters for non-linear regressions for fresh weight reduction for 2,4-D in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S).

Herbicide	Population	Slope	ED ₅₀	RF ₅₀	ED ₉₀	RF ₉₀
2,4-D	S	1.12 ± 0.01	30.63 ± 2.41	1	222.43	1.00
	R1	1.87 ± 0.03	68.35 ± 7.23 *	2.23	220.32	0.9
	R2	1.39 ± 0.2	94.48 ± 8.75 *	3.08	461.41	2.07
	R3	1.93 ± 0.16	75.95 ± 3.63 *	2.47	237.68	1.08
	R4	1.38 ± 0.17	94.62 ± 6.63 *	3.08	461.41	2.07
	R5	1.31 ± 0.16	88.84 ± 5.33 *	2.90	474.73	2.13
	R6	1.32 ± 0.14	106.41 ± 9.51 *	3.47	563.03	2.53

ED₅₀ and ED₉₀ represent the effective doses required for 50% and 90% reductions in plant biomass, respectively. RF₅₀ = resistance factor (ED₅₀ R/ED₅₀ S). RF₉₀ = resistance factor (ED₉₀ R/ED₉₀ S). The R² values range from 0.96 to 0.99, and the *p* values (probability level of significance of the non-linear model) are <0.0001. * = significant differences between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

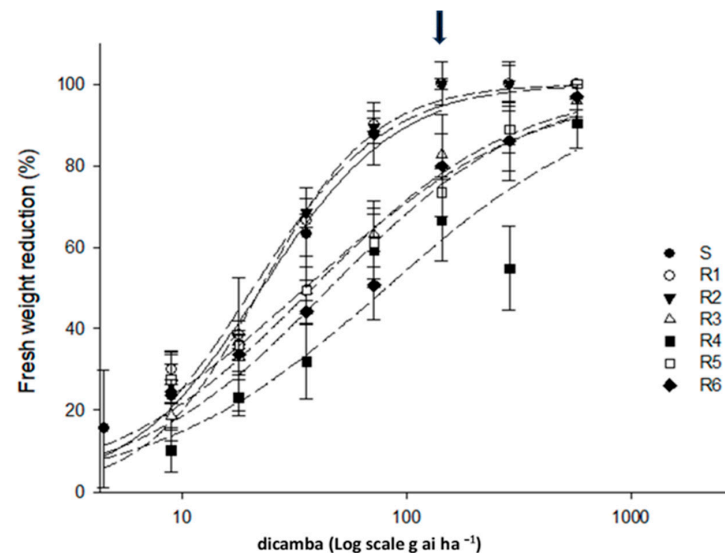


Figure 6. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for dicamba in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S). Solid line denotes the S population and short-dashed lines denote the R populations. The arrow denotes the recommended field rate of dicamba.

Table 9. Estimated parameters for non-linear regressions for fresh weight reduction for dicamba in *Papaver rhoeas* populations, R1, R2, R3, R4, R5, R6, and susceptible (S).

Herbicide	Population	Slope	ED ₅₀	RF ₅₀	ED ₉₀	RF ₉₀
dicamba	S	1.46 ± 0.16	22.76 ± 1.93	1	103.63	1.00
	R1	1.61 ± 0.22	21.63 ± 2.11 ns	0.95	102.65	0.95
	R2	1.72 ± 0.25	22.57 ± 1.85 ns	0.99	81.00	0.91
	R3	1.09 ± 0.09	39.18 ± 3.45 *	1.72	376.04	3.62
	R4	0.83 ± 0.01	81.23 ± 19.67 *	3.56	1154.44	11.14
	R5	0.96 ± 0.03	38.34 ± 5.56 *	1.68	379.60	3.66
	R6	1.23 ± 0.13	45.65 ± 6.23 *	2.00	437.61	4.12

ED₅₀ and ED₉₀ represent the effective doses required for 50% and 90% reductions in plant biomass, respectively. RF₅₀ = resistance factor (ED₅₀ R/ED₅₀ S). RF₉₀ = resistance factor (ED₉₀ R/ED₉₀ S). The R² values range from 0.95 to 0.98, and the *p* values (probability level of significance of the non-linear model) are <0.0001. ns = non-significant difference and * = significant differences between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

3.2.2. Malathion Effect

When malathion was applied alone at 1000 g ai ha⁻¹, there was no effect on growth of either the S or R populations. When 2,4-D was applied after malathion to the susceptible population, the behavior in terms of biomass was similar without the presence of the insecticide. In the presence of malathion, both R populations (R4 and R6) became susceptible to 2,4-D, and the RF for % of biomass reduction went down from 2.9 to less than 1 and from 3.36 to 1.62 (Figure 7, Table 10).

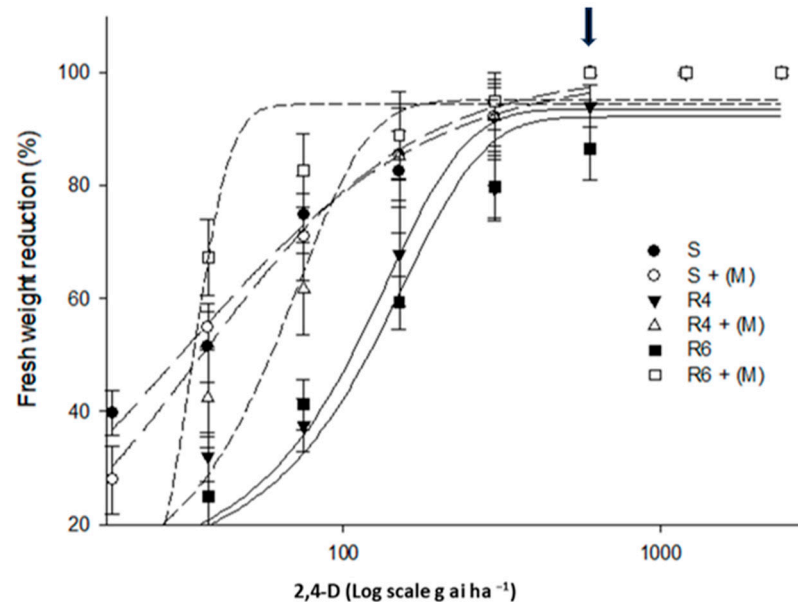


Figure 7. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for 2,4-D and 2,4-D plus malathion (M) at 1000 g ai ha⁻¹ in *Papaver rhoeas* populations, R3, R4, and susceptible (S). Long-dashed lines denote S population for imazamox and imazamox +M, solid line denotes the R populations for imazamox, and short-dashed lines denote the R populations for imazamox +M. The arrow denotes the recommended field rate of 2,4-D.

Table 10. Estimated parameters for fresh weight reduction for 2,4-D plus malathion (1000 g ai ha⁻¹) in *Papaver rhoeas* populations R3, R4, and susceptible (S).

Herbicide	Population	Slope	ED ₅₀	RF ₅₀
2,4-D	S	1.22 ± 0.21	30.63 ± 3.41	1
	+Malathion	1.24 ± 0.08	35.87 ± 1.74 ns	1.16
	R4	1.23 ± 0.15	89.11 ± 7.23	2.9
	+Malathion	1.12 ± 0.08	20.68 ± 1.82 *	-
	R6	1.19 ± 0.02	101.30 ± 6.05	3.36
	+Malathion	1.43 ± 0.12	48.81 ± 2.36 *	1.62

ED₅₀ = effective dose required for 50% reduction in plant biomass. RF = resistance factor (ED₅₀ R/ED₅₀ S). The R² values range from 0.92 to 0.98, and the *p* values (probability level of significance of the non-linear model) are <0.0001. ns = non-significant difference and * = significant differences between S and resistant populations by ANOVA (*p* < 0.05). ± denotes standard errors.

Significant differences in biomass reduction were observed in both (R) populations when treated with dicamba alone compared to dicamba combined with malathion. The ED₅₀ values decreased from 172.84 g ai ha⁻¹ to 28.08 g ai ha⁻¹ for the R4 population and from 102.40 g ai ha⁻¹ to 42.67 g ai ha⁻¹ for the R6 population when exposed to the herbicide-malathion combination (Figure 8, Table 11). Furthermore, when malathion was utilized in screening as a Cyt. P450 inhibitor, it substantially reduced the RF₅₀ from 7.8 to 1.27 for R4 and from 4.6 to 1.9 for R6. Remarkably, the S population showed no discernible

differences from the malathion application. The ED_{50} values remained relatively unchanged at approximately 222 g ai ha^{-1} .

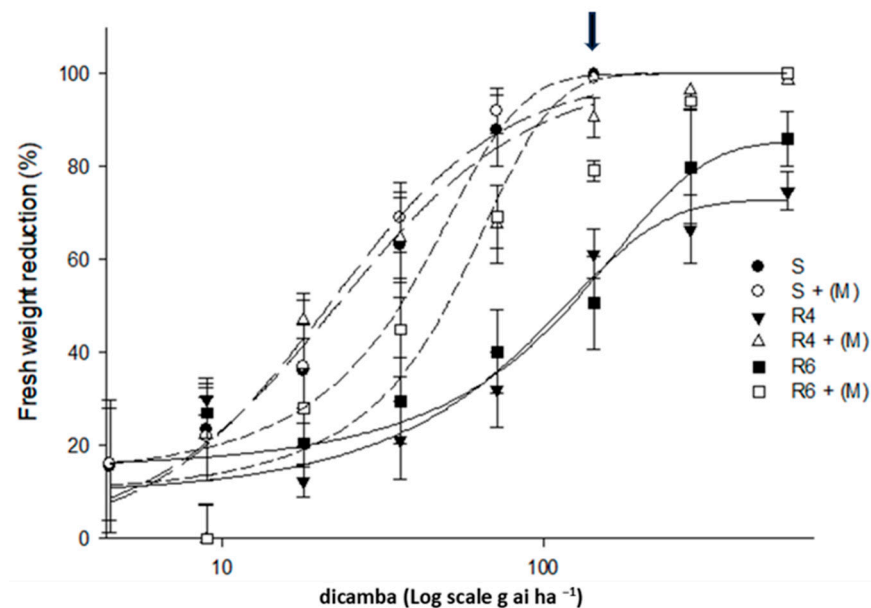


Figure 8. Dose–response curves represented in percentages of fresh weight reduction compared to untreated control for dicamba and dicamba plus malathion (M) at $1000 \text{ g ai ha}^{-1}$ in *Papaver rhoeas* populations, R3, R4, and susceptible (S). Long-dashed lines denote S population for imazamox and imazamox +M, solid line denotes the R populations for imazamox, and short-dashed lines denote the R populations for imazamox +M. The arrow denotes the recommended field rate of dicamba.

Table 11. Estimated parameters for fresh weight reduction for 2,4-D and 2,4-D plus malathion ($1000 \text{ g ai ha}^{-1}$) in *Papaver rhoeas* populations R3, R4, and susceptible (S).

Herbicide	Population	Slope	ED_{50}	RF_{50}
dicamba	S	1.45 ± 0.16	22.76 ± 1.93	1
	+Malathion	1.59 ± 0.22	$22.44 \pm 1.88 \text{ ns}$	1
	R4	1.72 ± 0.20	172.84 ± 11.36	7.8
	+Malathion	1.43 ± 0.35	$28.08 \pm 5.39 *$	1.27
	R6	1.02 ± 0.13	102.40 ± 13.05	4.6
	+Malathion	1.41 ± 0.18	$42.67 \pm 4.27 *$	1.9

ED_{50} = effective dose required for 50% reduction in plant biomass. RF = resistance factor ($ED_{50} \text{ R}/ED_{50} \text{ S}$). The R^2 values range from 0.95 to 0.99, and the p values (probability level of significance of the non-linear model) are <0.0001 . ns = non-significant difference and * = significant differences between S and resistant populations by ANOVA ($p < 0.05$). $\pm$ denotes standard errors.

4. Discussion

P. rhoeas is widespread throughout European countries and mainly infests winter cereal crops [24]. In Tunisia, winter wheat and barley (mainly rainfed) are also the most extended crops. However, there is an increasing number of control failure reports for ALS inhibitors and synthetic auxin herbicides to manage this weed species in these two winter cereals. This study confirms the presence of *P. rhoeas* populations from Tunisia with multiple resistance to ALS inhibitors and dicamba for the first time. Multiple resistance to these two sites of action is already reported in Mediterranean European countries, such as France, Greece, Italy, and Spain [5]. The range of herbicide doses used in this study in the entire dose–response curve adhered to the HRAC guidelines, which recommend using a spectrum of herbicide doses from sub-lethal to lethal levels for comparing resistant and susceptible weeds [2]. In our study, the lethal rate for all herbicides was achieved at four times the field rate, except for tribenuron-methyl, which, even at 64-fold the recommended

rate, did not achieve a 100% fresh weight reduction. Previous studies used the same range of doses for all herbicides for *Sinapis alba* [27], or up to 8 times the field rate for *P. rhoeas* [28]. In Greece, highly resistant populations to tribenuron-methyl showed resistance factors between 137 and over 2400 [29], while in Spain they observed between 2 and 695 [28], which was similar to this study in which we observed 77 to 263. These studies from Spain showed cross-resistance to florasulam and imazamox with RF₅₀ from 2 to 24 and 6 to 40, respectively, similar to the values in this research for imazamox of 4.9 to 11.5. Regarding synthetic auxins, previous studies found multiple R to 2,4-D [28,30] in contrast to this research, with moderate resistance confirmed only for dicamba, like others [28].

In this study, two populations (R4 and R6) were confirmed to have multiple resistance to tribenuron-methyl and dicamba based on their fresh weight reduction and malathion experiment, indicating that resistance is evolving in these two populations by means of non-target-site resistance mechanisms. Additionally, five R populations were cross-resistant to imazamox (R1, R2, R3, R4, and R6) and not to florasulam. However, R4 showed a different behavior compared to the susceptibility for this triazolopyrimidine with RF₉₀ around 4 indicating a suspected low cross-resistance to the three families of ALS-inhibiting herbicides tested. *P. rhoeas* biotypes with multiple resistance to different herbicides of ALS inhibitors and auxins (2,4-D and MCPA) were found in previous studies [22,24,30]. Different resistance mechanisms have been documented for both, encompassing TSR (mutation) and NTSR (enhanced metabolism) for ALS inhibitors. Additionally, two NTSR mechanisms have been identified for auxin mimics, comprising enhanced metabolism and reduced transport [31]. This indicates the presence of mechanistic diversity and remarkable genetics with different populations of *P. rhoeas*.

Regarding TSR mechanisms, all the R populations showed changes in position Pro197. This study found four different amino acid substitutions: Pro 197 His, Pro197Asn, Pro197Leu, and Pro197Thr. Pro197His was found in resistant poppy biotypes from the UK and conferred resistance to metysulfuron and tribenuron-methyl [16]. Pro197Thr was reported in a previous study in Italian populations [21]. Other mutant ALS alleles were found in a previous study, namely Pro197Arg, Pro197Leu, Pro197Ala, and Pro197Ser [24,31,32]. In particular, the substitution of Pro by Ser is reported in several European countries as conferring high levels of resistance to tribenuron-methyl in *P. rhoeas* [19,21]. Recently, in resistant *P. rhoeas* biotypes from Portugal, Pro197Phe has been identified as a double-nucleotide substitution conferring resistance to sulfonylureas and imidazolinones [30]. A study showed that the substitutions Pro197Leu and Pro197Ser were the most frequent while Pro197Ala was present at a lower frequency. Trp574Leu was not found in our study. However, it was identified previously [18] and the mutation in this codon has been found less frequently [24,33].

Regarding NTSR mechanisms, enhanced metabolism was found in the two R populations investigated, R3 and R4, presenting Pro197Thr and Pro197Thr plus Pro197Leu, respectively. The results from dose-response experiments using the Cyt. P450 inhibitor malathion together with imazamox support that both populations are able to degrade imazamox. Metabolism studies have confirmed the presence of enhanced metabolism in Spanish populations of *P. rhoeas* to imazamox [14] and to tribenuron-methyl in a Portuguese population [30]. When considering the R3 population, where resistance may be attributed solely to NTSR mechanisms, definitive conclusions regarding the R4 population remain elusive regarding the relative contribution of both TSR and NTSR mechanisms because they can coexist within the same population or even within individual plants [17,18]. The NTSR mechanisms of ALS-inhibiting herbicides might be underestimated in broadleaved weeds because it is frequently associated only with TSR [18]. Moreover, NTSR effects are often observed at the level of individual plants and accumulate gradually over generations [17].

In this research, all populations were susceptible to 2,4-D, as they died at double the field rate. On the other hand, low resistance to dicamba was confirmed in two populations (R4 and R6), while for the other R populations, this was less evident. This is not aligned with the majority of reports that include *P. rhoeas* biotypes resistant to 2,4-D [5], which sometimes can be cross-resistant to dicamba [24]. Experiments with malathion showed synergism,

indicating the presence of P450-mediated enhanced metabolism. In the R populations, the resistance to dicamba can apparently be conferred by NTSR mechanisms in *P. rhoeas*, particularly enhanced metabolism. Up to now, two different NTSR mechanisms had been found in *P. rhoeas*, specifically reduced transport and enhanced metabolism [29]. Both mechanisms were observed in previous studies on auxins in R populations from Spain. The relationship between impaired translocation and enhanced metabolism remains unclear, particularly in ascertaining whether they are related or independent mechanisms [11,22].

5. Conclusions

In the evaluation of the resistance of *P. rhoeas* to ALS inhibitors and auxins, multiple resistance was found in populations from Tunisia. All R populations showed resistance to tribenuron-methyl with five showing cross-resistance to imazamox. Additionally, one population demonstrated potential cross-resistance to florasulam as well. In five out of six R *P. rhoeas* populations, TSR by mutations was identified in codon 197 of the *ALS* gene, namely, Pro197His, Pro197Thr, Pro197Leu, and Pro197Asn. Experiments using the P450 inhibitor malathion together with imazamox showed a reduction in resistance factors within the two populations tested. In the R population, TSR and NTSR to ALS inhibitors likely coexist. Regarding auxins, moderate resistance to dicamba and no resistance to 2,4-D were confirmed. The most feasible and proposed explanation for dicamba is the presence of NTSR via enhanced metabolism. The diversity of resistance mechanisms uncovered in this study underscores the high genetic variability and adaptability of *P. rhoeas*. The rotation or combination of different herbicide modes of action is effective in preventing or delaying the emergence of resistance [34]. A strategy based on proactive and reactive measures in terms of integrated weed management, focusing on non-chemical and cultural strategies, should be implemented to reduce the development of herbicide resistance in *P. rhoeas* in Tunisia.

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