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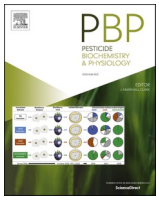

# Tribenuron-methyl metabolism and the rare Pro197Phe double mutation together with 2,4-D metabolism and reduced absorption can evolve in *Papaver rhoeas* with multiple and cross herbicide resistance to ALS inhibitors and auxin mimics

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ABSTRACT

Multiple resistance mechanisms to ALS inhibitors and auxin mimics in two *Papaver rhoeas* populations were investigated in wheat fields from Portugal. Dose-response trials, also with malathion (a cytochrome P450 inhibitor), cross-resistance patterns for ALS inhibitors and auxin mimics, alternative herbicides tests, 2,4-D and tribenuron-methyl absorption, translocation and metabolism experiments, together with ALS activity, gene sequencing and enzyme modelling and ligand docking were carried out. Results revealed two different resistant profiles: one population (R1) multiple resistant to tribenuron-methyl and 2,4-D, the second (R2) only resistant to 2,4-D. In R1, several target-site mutations in Pro197 and enhanced metabolism (cytochrome P450-mediated) were responsible of tribenuron-methyl resistance. For 2,4-D, reduced transport was observed in both populations, while cytochrome P450-mediated metabolism was also present in R1 population. Moreover, this is the first *P. rhoeas* population with enhanced tribenuron-methyl metabolism. This study reports the first case for *P. rhoeas* of the amino acid substitution Pro197Phe due to a double nucleotide change. This double mutation could cause reduced enzyme sensitivity to most ALS inhibitors according to protein modelling and ligand docking. In addition, this study reports a *P. rhoeas* population resistant to 2,4-D, apparently, with reduced transport as the sole resistance mechanism.

## 1. Introduction

Weeds cause significant crop losses by competing for nutrients, water or light (Oerke, 2006). While non-chemical weed control methods can be effective (Bond and Grundy, 2001), synthetic herbicides are still the most widespread weed control tool in modern agriculture (Délye et al., 2013). However, their widespread use has raised concerns about impacts on the environment and human health (Annett et al., 2014). Moreover, a major consequence of the excessive reliance on weed control mostly or exclusively based on herbicides and the intensive use of a limited number of herbicide sites of action (SoAs) has been the development of herbicide resistance (Moss, 2004).

*Papaver rhoeas* L. is the best-known weed species of the genus *Papaver*, which is represented in the *Papaveraceae* family (Holm et al., 1997). *P. rhoeas* is an annual, broadleaf cosmopolitan species that is widespread across Europe, predominantly in central and southern

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regions, with lower frequencies in northern Europe (Délye et al., 2011). *P. rhoeas* frequently has been reported as a common weed of winter wheat (*Triticum aestivum* L.) and other winter cereals (Mitich, 2000), and if left unmanaged can substantially reduce yields (Rey-Caballero et al., 2017a; Wilson et al., 1995). Its high fecundity, extended period of emergence, and seed longevity generate a persistent soil seed bank and give this weed a high potential to invade and spread in cultivated areas (Cirujeda et al., 2008; Lutman et al., 2002). Chemical control of *P. rhoeas* relies heavily on post-emergence herbicides, mainly acetolactate synthase (ALS) inhibitors (WSSA/HRAC Group 2/B), auxin mimics (WSSA/HRAC Group 4/O), and, to a lesser extent, photosynthesis II inhibitors (WSSA/HRAC Groups 5 and 6) (Torra et al., 2010).

The auxin mimic herbicide family is classified in five chemical groups, containing pyridine-carboxylates (i.e. aminopyralid), quinoline-carboxylates (i.e. quinclorac), benzoates (i.e. dicamba), pyridyloxy-carboxylates (triclopyr and fluroxypyr) and phenoxy-carboxylates (i.e. 2,4-D) (Busi et al., 2018). 2,4-D (2,4-dichlorophenoxyacetic acid) was the first herbicide belonging to this group to be commercially developed and released worldwide in 1945 (Todd et al., 2020). 2,4-D supplied very powerful management tool for the majority of broadleaved weed species in cereals, as a result of its low cost, selectivity, efficacy, and extensive spectrum of weed management (Mithila et al., 2011). 2,4-D kills plants as a result of excessive auxin signaling that leads to abnormal cell division, high levels of ROS production and activation of senescence and plant death by ethylene production (Song, 2014). Moreover, the efficacy of 2,4-D as herbicide depends on numerous factors such as uptake and translocation to the plant meristems (Torra et al., 2017). Although auxin mimic herbicides had been on the market for nearly 70 years, globally, there are 82 reported resistant cases, including 42 different species, 25 of which, thus far, are resistant to 2,4-D herbicide (Heap, 2022). Target site resistance (TSR) to auxin mimic herbicides has recently been reported in two cases (LeClere et al., 2018; Figueiredo et al., 2022), while non-target site resistance (NTSR) mechanisms, which consist of decreased herbicide uptake, reduced translocation, and detoxification, have been reported as the putative resistance mechanisms in more weeds (Busi et al., 2018; Mithila et al., 2011). Enhanced detoxification and impaired transport were reported as resistance mechanisms to 2,4-D in *P. rhoeas* (Rey-Caballero et al., 2016; Torra et al., 2017). Besides the cytochrome P450 monooxygenase (P450) inhibitor malathion restored 2,4-D activity or ceased metabolism in herbicide resistant *P. rhoeas* (Torra et al., 2017; Torra et al., 2021a).

The ALS inhibitors are categorized into six chemical families, sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-benzoates (PYB), triazolinones (TZ) and sulfonanilides (Shaner, 2014). They have proved to be the herbicide SoA at most risk to provoke the evolution of resistance (Tranel and Wright, 2002), with resistance cases reported in 170 weed species to date (Heap, 2022). Tribenuron-methyl is an active substance of the SU family, which has been available on European markets since 1986 (Cirujeda et al., 2001). This herbicide inhibits the biosynthesis of branched amino acids (valine, leucine, and isoleucine) by blocking substrate access to the active site of ALS enzyme (Rey-Caballero et al., 2017b). Resistance to ALS-inhibitors is frequent because certain mutation(s) in the *ALS* gene cause changes in the protein that reduce the affinity of the ALS enzyme for herbicides (Kaloumenos et al., 2009; Powles and Yu, 2010), and in fewer cases because of enhanced rates of herbicide metabolism, particularly in broadleaved weeds (Hatami et al., 2016; Hada et al., 2021). In *P. rhoeas*, resistance to ALS inhibiting herbicides has usually been explained by TSR attributed to different mutations in the *ALS* gene (Délye et al., 2011; Kaloumenos et al., 2009; Marshall et al., 2010; Rey-Caballero et al., 2017b). Nevertheless NTSR, i.e. enhanced metabolism, has also been reported more recently (Rey-Caballero et al., 2017b; Scarabel et al., 2015). All these mechanisms can confer resistance to ALS inhibiting herbicides in this species, coexisting within a single population (Scarabel et al., 2015), and even the same plant (Rey-Caballero et al., 2017b).

The increase in both monoculture farming and overuse of 2,4-D followed by tribenuron-methyl application have selected ALS and/or 2,4-D herbicide-resistant *P. rhoeas* biotypes (Rey-Caballero et al., 2016). Multiple resistance is certainly considered one of the largest issues that resistance provides since it makes it hard to select alternative herbicides, especially while numerous resistance mechanisms are observed within the same population (TSR and NTSR) (Torra et al., 2021b). Resistance to ALS inhibitors and auxin mimic herbicides has been stated in populations from 15 species so far, such as *P. rhoeas* (Heap, 2022). In *P. rhoeas*, multiple herbicide resistance to both SoAs has been reported in Spain, Greece, Italy and France (Heap, 2022). Kati et al. (2019) confirmed the prevalence of multiple resistance to ALS inhibitors and to auxin mimics in individual *P. rhoeas* plants in those countries. The fact that *P. rhoeas* is a self-incompatible species leads to obligatory recombination of genetic factors (Délye et al., 2013), and probably this reason has driven the accumulation inside individual plants of various resistance mechanisms found in a population. A better understanding of resistant mechanisms in *P. rhoeas* may also improve resistance management by better defining herbicide use patterns to delay or avoid resistance to these SoAs. This study was thus conducted in order (1) to confirm the resistance of *P. rhoeas* to 2,4-D and tribenuron-methyl and (2) to investigate the TSR and NTSR mechanisms involved in the resistant *P. rhoeas* populations harvested from wheat fields in Portugal.

## 2. Material and methods

### 2.1. Plant material

The susceptible seeds (S) of *P. rhoeas* used were researched in previous studies (Torra et al., 2017). The resistant (R) population R1 evolved in the field at label doses of 2,4-D (600 g ai $ha^{-1}$) and tribenuron-methyl (TM) at 20 g ai $ha^{-1}$ in Beja (38° 02′ 06" N 7° 53′ 26" W) in central Portugal (Alentejo), and seeds were collected during 2020 as a putative multiple R. Additionally, a second population R2 suspected of being R only to 2,4-D was also studied, this last population was harvested in the same region (38° 00′27"N 7° 52′ 20"N), also in 2020.

### 2.2. Chemicals

The commercial herbicides used in this study to determine the physiological effects on the S, R1 and R2 populations of *P. rhoeas* are shown in Table 1. To carry out the absorption and translocation experiments $^{14}$C-2,4-D acid (DuPont de Nemours & Co, Nambsheim, France) and $^{14}$C-TM (American Radiolaoted Chemicals, Inc., Saint Louis, MO, USA) were used. The analytical grade (> 99.5%) was used to determine herbicidal effects in physiological and biochemical studies in plants.

### 2.3. Dose-response assays and malathion effect

Mature seeds of *P. rhoeas* were germinated in Petri dishes with distilled water and filter paper, placed in a growth chamber at 28/18 °C (day / night), 16 h photoperiod, 80% relative humidity and a photosynthetic photon flux of 850 μmol $m^{-2}$ $s^{-1}$. The seedlings of all populations were individually transplanted into plastic pots (448 $cm^3$) containing sand / peat in a 1: 2 (v / v) ratio, and then placed in a greenhouse at 28/18 °C (day / night). Plants were treated with herbicides at the 4-leaf stage. Herbicide treatments were carried out in a laboratory chamber sprayer (SBS-060 De Vries Manufacturing, Hollandale, MN, USA), with a 8002 E flat fan nozzle delivering 200 L $ha^{-1}$ at 250 kPa at 50 cm height. The different doses of 2,4-D and TM used are shown in Table S1 of the Supporting Information. The survival of the plants was recorded at 28 days after the treatment (DAT). Also, the plants were harvested at ground level and weighed to determine their dry weight (60 °C for four days). Both dry weight and survival data were converted to percentages compared to untreated control plants. The experiment was repeated three times with five repetitions per combination of treatment and population.

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

| Herbicide | Company | Commercial Product | MOA (HRAC) | Field Dose (g ai ha$^{-1}$) |
|---|---|---|---|---|
| **Bispyribac-sodium**[a] | Bayer CropScience | 40.8% *w/v*, Nominee® SC | ALS inhibitor | 40 |
| **Florasulam** | FMC | 5% w/v, Fragma® SC | ALS inhibitor | 5 |
| **Flucarbazone** | Arysta LifeScience | 70% *w/w*, EVEREST® WG | ALS inhibitor | 25 |
| **Imazamox** | BASF | 4% w/v, Pulsar® 40 SC | ALS inhibitor | 40 |
| **TM** | Nufarm | 75% w/v, Primma® SL | ALS inhibitor | 20 |
| **2,4-D** | Nufarm | 60% w/v, U 46 D Complet® SL | Auxin mimics | 600 |
| **Clopyralid** | Corteva | 72% w/v, Lontrel® WG | Auxin mimics | 300 |
| **Dicamba** | Syngenta | 48% w/v, Banvel® D WG | Auxin mimics | 144 |
| **Fluroxypyr** | Nufarm | 20% *w/v*, Praxis®. EC | Auxin mimics | 200 |
| **MCPA** | Probelte | 40% w/v, Procer M-40® | Auxin mimics | 1000 |
| **Fomesafen** | Syngenta | 25% w/v, Flex® SL | PPO inhibitor | 375 |
| **Glyphosate** | Bayer CropScience | 36% w/v SL, Roundup® | EPSPS inhibitor | 720 |
| **Metribuzine** | Bayer CropScience | 60% w/v, Sencor® SC | PS II inhibitor | 60 |
| **Mesotrione** | FMC | 10 w/v, Border® SC | HPPD inhibitor | 150 |

[a] +BioPower (0.6 L ha$^{-1}$).

Malathion has previously been shown to inhibit 2,4-D and ALS inhibitors metabolism in R *P. rhoeas* by inhibiting P450 (Torra et al., 2021a). Plants from populations R1 and R2 were treated with malathion at 1000 g ai ha$^{-1}$ in the 4-leaf stage and then dried for 1 h at laboratory temperature (24 °C). After that, 2,4-D (R1 and R2) and TM (R1) were applied separately at the doses shown in Table 2. The experiments were repeated twice with three repetitions (ten technical repetitions for each R population).

### 2.4. Cross and multiple-resistance herbicides

To evaluate the efficacy of alternative chemical options, cross resistance between ALS inhibitors and auxin mimics families, and potential cross and multiple resistance to other SoAs, different herbicides were applied (under the same conditions and spray volume as the previous trial) on *P. rhoeas* S, R1 and R2. populations. The diverse herbicides and field doses used are shown in Table 1. The plants were cut 28 DAT, after which the fresh weight and plant survival percentage values of the plants were determined. The treatments were replicated three times in a completely randomized design, using 10 plants per dose and population. The experiment was repeated twice.

### 2.5. $^{14}$C-2,4-D and $^{14}$C-TM absorption and translocation assays

The $^{14}$C-2,4-D and $^{14}$C-TM absorption and translocation tests were carried out separately as described by Palma-Bautista et al. (2020) and Cruz-Hipolito et al. (2013), respectively. The $^{14}$C-2,4-D was mixed with a commercial formulation, with a final concentration of 600 g ai ha$^{-1}$ in 250 L ha$^{-1}$ with a specific activity of 0.834 kBq μL$^{-1}$. For the $^{14}$C-TM experiment, the final concentration was 20 g ai ha$^{-1}$, and the same specific activity. Five plants per time (96 HAT) and population were used with each herbicide. The sample radioactivity was measured for 10 min by LSS. The radioactive absorption and translocation values of $^{14}$C were expressed as a percentage of the total herbicide of $^{14}$C applied and recovered, respectively (Rey-Caballero et al., 2016; Rey-Caballero et al., 2017b).

### 2.6. 2,4-D metabolism studies

The plants of the populations S, R1 and R2 in the 4-leaf stage were treated with doses of commercial 2,4-D, at 0 and 400 g ai ha$^{-1}$ as described above for the first plant resistance experiments whole (Palma-Bautista et al., 2020). Five plants were collected from each population and dosed at 96 HAT. The plants were separated into the aerial part (leaves and shoots) and the root system and were subsequently rinsed with distilled water to eliminate the unabsorbed 2,4-D herbicide. They were snap frozen in liquid nitrogen and then stored at −80 °C. For these trials, the methodology described by Mora et al. (2019) and Palma-Bautista et al. (2020) continued to use a Beckman Coulter Gold LC system (Fullerton, CA, USA) equipped with a diode array detector (wavelength range 190–600 nm). Chromatographic separation was carried out using a Kinetex® EVO C18 column (150 mm, 4.6 mm ID, 2.6 μm particle size) from Phenomenex Inc. (Torrance, CA, USA), Equipped with 4.6 mm SecurityGuard TM ULTRA cartridges. The quantification of 2,4-D and its non-toxic metabolites was based on the 2,4-D calibration curve. The results were expressed as a percentage of 2,4-D and its metabolites. The experiment was performed for each resistant and susceptible population and was repeated twice at different times in the laboratory under similar climatic conditions.

### 2.7. Effect of TM on ALS activity

This study was carried out to quantify the ALS activity in *P. rhoeas* populations following the methodology described by Hatami et al. (2016). Plant tissue from S, R1 and R2 populations was used for the assays and grown under the conditions described previously. Leaf tissues (3 g) from plants at the 4-leaf stage were frozen. The protein was determined by the Bradford method (Bradford, 1976). The herbicide concentration required to inhibit ALS activity by 50% ($I_{50}$) was calculated (Hatami et al., 2016; Rosario et al., 2011). Three experiments, each performed with a separate tissue extract from a different plant, were conducted for each population, and each sample at each herbicide concentration was assayed in triplicate.

### 2.8. ALS gene sequencing assays

#### 2.8.1. DNA isolation

In this step, plants from S, R1 and R2 were grown as mentioned in the plant growing section. A total of 12 plants per population were selected and applied with field dose of TM (20 g ia ha$^{-1}$) in Iberian Peninsula. Four days before the application, 500 mg of young foliar tissue was harvested from each plant. The samples were placed in liquid nitrogen (N2), then were lyophilized (LyoQuest, H140 BeiJer, Telstar, Terrassa, Barcelona) at −50 °C and between 10 and 0.03 mBar for 48 h. Approx., 20 mg of lyophilized tissue was used for DNA extraction. This step was performed using the Speedtools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Valle de Tobalina, Madrid, Spain). The concentration of each sample was measured in a NANODROP Thermoscientific spectrophotometer (ThermoFisher, Nano-Drop Products, Wilmington, DE).

#### 2.8.2. PCR amplification of conserved domains

Two sets of primers were used to amplify the conserved domains. Domains C, A and D were amplified with forward Up02 5'-GAAACACCCATTTCCACCAC-3′ and reverse Down02 5'-TGGGTGCTCGAGACATATACC-3′ (Kaloumenos et al., 2009). On the other hand, domains B and E were amplified with forward (PaF) 5'-CTGCCGTTGCTAAACCTGA-3′ and reverse (PaR) 5'AAGACCTAGCAAGCTGAGAGAC-3′. The PCR conditions were the same for the two sets of primers except for annealing temperature (supplementary material 1).

#### 2.8.3. Gene sequencing

PCR amplification product were visualized under ultraviolet light (320 nm; ALPHA DIGI DOC Pro instrument, Alpha Innotec Corporation, Johannesburg, South Africa). Then, bands of amplicons were cut and the purified using the Speed Tools PCR Clean-Up Kit (Biotools, B&M Labs, Madrid, SpainSamples were sequenced using an external lab (STABVIDA, Caparica, Portugal). The sequencing results were visualized using CHROMAS software. After that, these sequences were aligned using the CLUSTAL OMEGA software.

### 2.9. ALS enzyme modelling and ligand docking

*P. rhoeas* ALS enzyme (PrALS) nucleotide sequence was obtained from NCBI (AJ577316). We used the protein structure homology-modelling server, SWISS-MODEL (Waterhouse et al., 2018) to generate a PrALS model using the crystal structure of *Arabidopsis thaliana* ALS (PDB 3E9Y) as template. We used the same server for the structural assessment of the PrALS model. We used Glide (Schröedinger) for in silico docking of the herbicides TM and imazamox. The different PrALS amino acid changes were modeled using SWISS-MODEL and minimized using Maestro (Schröedinger). Docking results and protein models were visualized with Chimera (Pettersen et al., 2004).

### 2.10. Statistical analyses

The experiments followed a completely randomized design with three replicates per treatment. The experimental unit was comprised of one pot containing four plants. Analyses of dose-response data in the whole plant and ALS activity assays were performed using the R software (drc package) by fitting the data to a nonlinear log logistic regression model as follows:

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

Where d is the upper limit, c is the lower limit, b is the slope at the inflexion point, g denotes $GR_{50}$ (or $LD_{50}$ and $I_{50}$) and x is the independent variable (herbicide dose). When the c parameter was not significant with 0, it was deleted from the equation, using three parameter logistic functions. The plant dry weight and ALS activity were taken relative to the untreated control. The resistance factor (RF) was calculated by dividing the determined $GR_{50}$ (or $LD_{50}$ and $I_{50}$) value of the resistant population by that of the sensitive population. The effect of population and the time, as well as their interaction in the $^{14}$C-tribenuron methyl ($^{14}$C TM) absorption, were subjected to an ANOVA. The population was the fixed factor and the time the random factor. The means and standard errors of $^{14}$C-TM absorption and translocation were calculated for all plant parts, and the means were analyzed by different groups. Assumptions of equal variance and normal distribution were evaluated. The LSD test at 5% probability was used for mean separation when necessary. Statistical analyses were performed using the Statistix (Analytical Software, United States of America) software (version 9.0).

## 3. Results

### 3.1. Dose-response assays and malathion effect

The R1, R2 and S *P. rhoeas* populations had a general common behavior after the first days of 2,4-D application: appearance of epinasty, growth reduction and morphological damage at high doses (≥640 g ai ha$^{-1}$) in the R1 and R2 populations and at low doses (40 g ai ha$^{-1}$) in the S population. At field doses (640 g ai ha$^{-1}$), only the resistant plants R1 and R2 survived the application of the herbicide. Resistance to 2,4-D was 40 higher in R1 and 7.5 in R2 compared to the high sensitivity found in the S population (Table 3 and Fig. 1). The studies carried out with these three populations showed that only the R1 population survived the field dose of TM used by farmers in the area (20 g ai ha$^{-1}$). The R1 population showed an RF of 86 times higher than the R2 and S populations. This indicates the multiple resistance to 2,4-D and TM in the R1 population but not in the R2 population, which only presented resistance to the synthetic auxin (Table 3 and Fig. 1).

In our studies we observed that malathion inhibited the high resistance of the R1 population to 2,4-D, lowering the $GR_{50}$ values of 512 without malathion to 74 with the application of the P450 inhibitor. However, the R2 population did not suffer any effect from the application of malathion, observing that the $GR_{50}$ values remained similar. Effect of malathion was also observed on the resistance of the R1 population of *P. rhoeas* to TM reducing the value of $GR_{50}$ from 51.4 to 34.6 (without/with malathion, respectively) (Table 3 and Fig. 1). These results indicate that metabolism is implicated in the resistance to 2,4-D and TM herbicide in the R1 population.

### 3.2. $^{14}$C-2,4-D and $^{14}$C-TM absorption and translocation assays

There were no significant differences ($P > 0.05$) between the R1, R2 and S populations in the herbicide absorption into the leaf tissue at 96 HAT for both herbicides (Fig. 2). However, absorption of $^{14}$C-2,4-D in both sensitive and R populations was 1.8 times greater than $^{14}$C-TM case (Fig. 2). The $^{14}$C-herbicides moved from treated leaf to the remainder shoots and roots, however, the behavior of both herbicides was different. Thus, in the case of 2,4-D, the herbicide moves in greater quantity towards the shoots and roots system in the S population than in the R1 and R2 populations of *P. rhoeas*. On the contrary, there were not significant differences in translocation of $^{14}$C-TM between S and R populations (Fig. 2).

**Table 3**
Parameters of the log-logistic equations a used to calculate the 2,4-D and TM rates (g ai ha$^{-1}$) with or without a previous malathion treatment required for 50% reduction dry h weight ($GR_{50}$) or survival ($LD_{50}$), of *P. roheas* populations from Southern Portugal harvested in cereal crops.

| Herbicide | Population | b | d | $GR_{50}$ | 95% CI | | RF | b | d | $LD_{50}$ | 95% CI | | RF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | | | Lower | Upper | |
| 2,4-D | R1 | 1.4 | 101.5 | **511.6** | 420.2 | 603.3 | 40.0 | 5.2 | 100.1 | **1672.7** | 1502.1 | 1843.2 | 17.4 |
| | + malathion | 1.1 | 99.4 | **73.8** | 54.1 | 93.5 | 5.8 | 5.8 | 100.0 | **530.3** | 498.0 | 562.7 | 6.1 |
| | R2 | 0.8 | 98.4 | **96.2** | 62.2 | 130.3 | 7.5 | 4.3 | 99.6 | **777.8** | 703.0 | 852.6 | 8.1 |
| | + malathion | 0.8 | 98.9 | **92.1** | 47.3 | 96.8 | 5.6 | 4.0 | 99.7 | **745.7** | 708.6 | 782.9 | 8.6 |
| | S | 0.8 | 95.2 | **12.8** | 8.2 | 17.3 | – | 5.9 | 100.1 | **96.4** | 86.0 | 106.9 | – |
| | + malathion | 0.8 | 98.2 | **12.8** | 8.3 | 16.8 | – | 6.6 | 100.0 | **86.5** | 64.9 | 108.1 | – |
| TM | R1 | 1.3 | 97.1 | **51.4** | 41.3 | 61.4 | 85.6 | 5.2 | 99.9 | **147.7** | 136.7 | 158.7 | 41.0 |
| | + malathion | 1.1 | 97.1 | **34.6** | 28.6 | 39.7 | 69.2 | 4.5 | 100.0 | **137.2** | 126.6 | 147.7 | 85.7 |
| | R2 | 1.0 | 98.9 | **0.8** | 0.6 | 1.0 | 1.3 | 4.0 | 99.8 | **4.7** | 4.3 | 5.0 | 1.3 |
| | + malathion | 1.0 | 98.9 | **0.7** | 0.6 | 0.9 | 1.4 | 2.9 | 101.8 | **2.2** | 2.0 | 2.4 | 1.4 |
| | S | 1.2 | 99.3 | **0.6** | 0.4 | 0.7 | – | 10.5 | 100.0 | **3.6** | 2.1 | 5.1 | – |
| | + malathion | 1.3 | 99.4 | **0.5** | 0.4 | 0.7 | – | 2.4 | 99.7 | **1.6** | 1.4 | 1.8 | – |

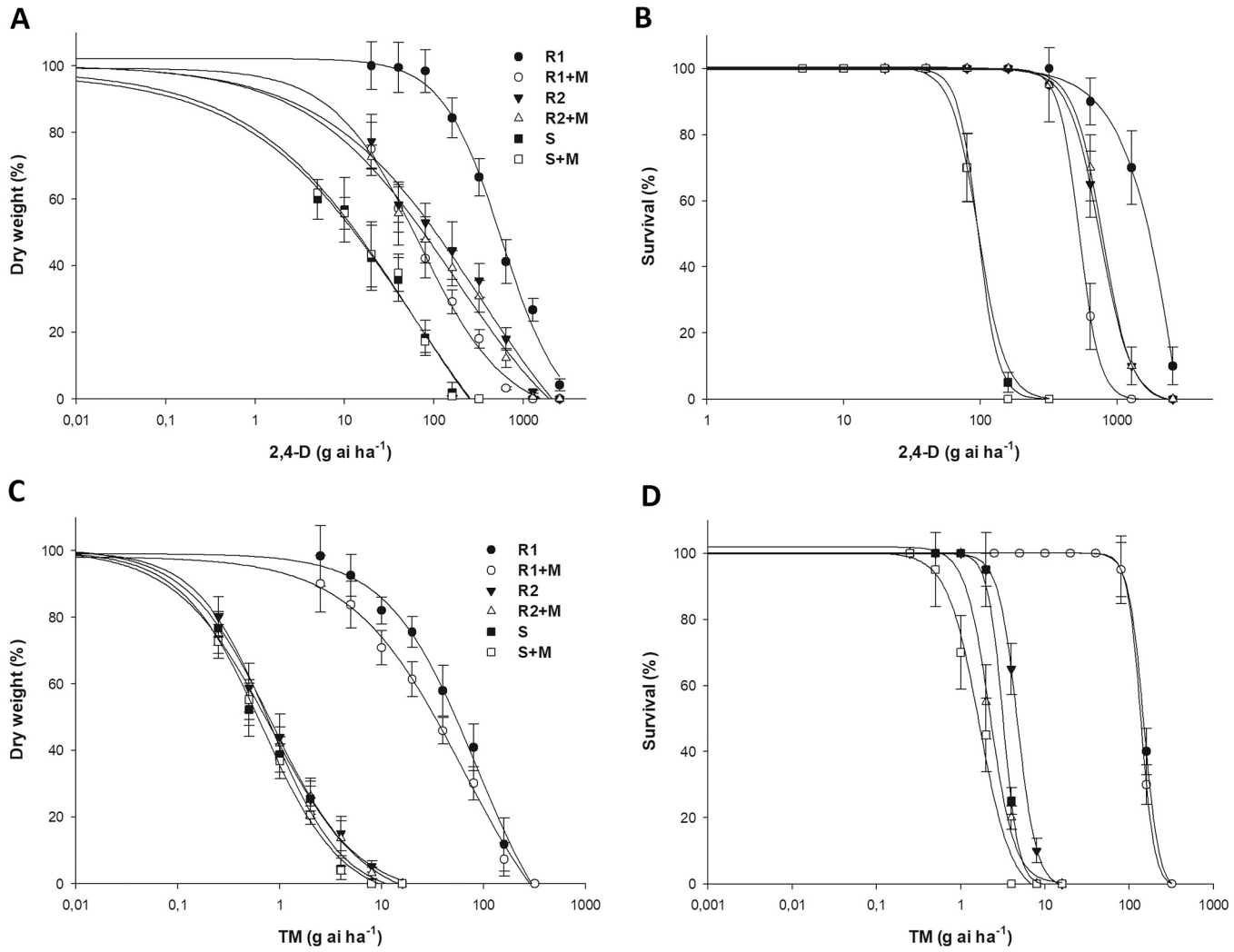


**Fig. 1.** Dose-response curves represented in percentage dry weight reduction and survival treated without and with malathion (1000 g ai ha$^{-1}$) for herbicides (A-B) 2,4-D and (C-D) TM in *P. rhoeas* populations (R1, R2 and S).

### 3.3. 2,4-D and TM metabolism studies

Qualitative and quantitative differences were found between the three populations both for 2,4D and TM. For 2,4-D, putative non-toxic metabolites were only detected in population R1 at 96 HAT (Fig. 3). In this population, >60% of 2,4-D was metabolized and only 35% of herbicide remained. Conversely, in S and R2 populations >90% of the herbicide was not degraded and <10% of 2,4-D was converted to putative non-toxic metabolites.

For TM, the three metabolites (metsulfuron-methyl (MM), metsulfuron-methyl-hydroxylate (OH-MM), and conjugate-MM) were detected at 96 HAT only in R1 population, while in the S and R2 populations only one toxic-metabolite (MM) was found (Fig. 3). With respect to the amount of the different metabolites between the populations, the amount of the $^{14}$C-TM found in R2 and S plants was higher than in R1 plants (Fig. 3). The *N*-demethylation of TM to form MM (active sulfonylurea herbicide) was similar in all plants and there were no significant differences. The MM metabolite was rapidly degraded, first through the hydroxylation of the phenyl ring generating the OH-MM, which was not present in S and R2 plants, but was in the R population R1 much higher than the first one (Fig. 3). The third metabolite, conjugate-MM, was formed by conjugation of OH-MM with carbohydrates and only appeared in the R1 population. Both OH-MM and conjugate-MM are non-toxic for plants (Cruz-Hipolito et al., 2013) and due to the differences found in the amounts of metabolites between the populations, resistance of R1 plants was likely based on increased TM metabolism.

### 3.4. Effect of TM on ALS activity

The RF for the R1 population treated with TM was 36.9 times higher than the S *P. rhoeas* population, while no difference was found between the R2 and S populations (Supporting Information: Table S2 and Fig. S1). The in vitro specific activity of the ALS enzyme obtained from the R1, R2 and S shoots of plant tissue was similar, without significant differences (395, 404 and 411 nmol of acetoin per mg of protein per hour, respectively).

### 3.5. ALS gene sequencing assays

No substitutions at codons corresponding to Pro197 and Trp574 were found in plants from S and R2 populations (Table 4). Also, no mutations were found for Trp574 in R1 population. However, in this population, four amino-acid replacements were identified at amino acid Pro197 (Phe197, Leu197, Ser197 and Thr197). Six different genotypes were identified in R1 population, being Pro197Ser and Pro197Phe the most abundant amino-acid substitutions (Table 4).

The sequencing chromatograms of *ALS* gene highlighting the amino

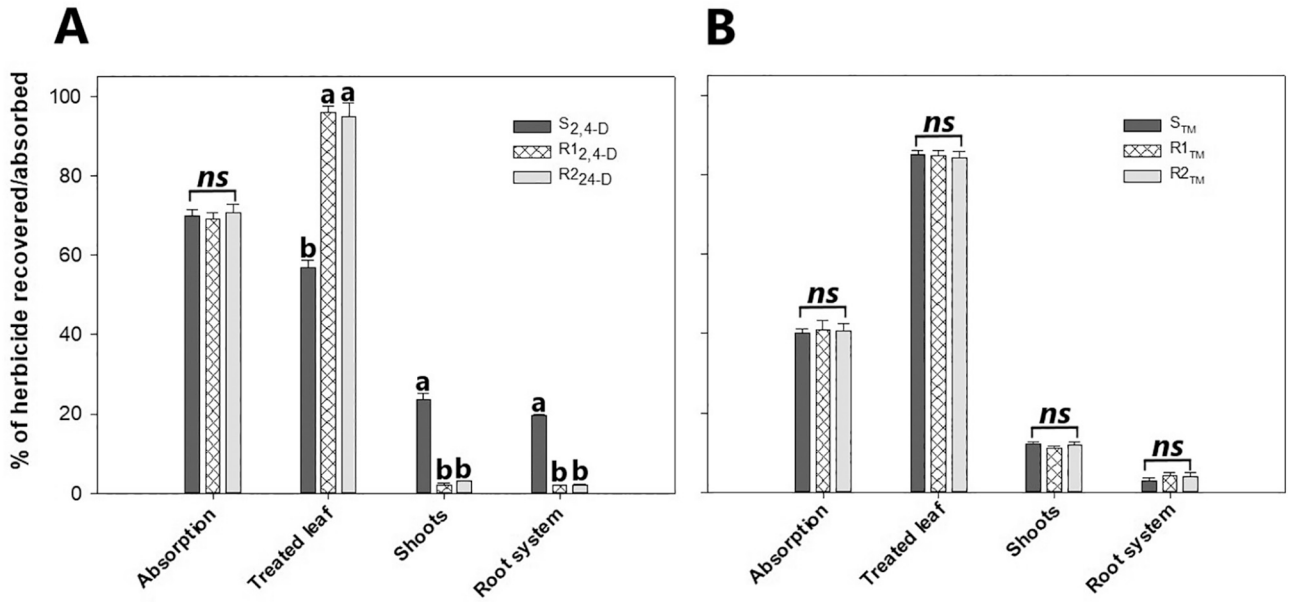


**Fig. 2.** Absorption (percent recovered radioactivity) and translocation (percent of absorbed radioactivity) of A) $^{14}$C-2,4-D (400 g ai ha$^{-1}$) and B) $^{14}$C-Tribenuron-methyl (TM) (20 g ai ha$^{-1}$) in S, R1 and R2 populations of *P. rhoeas* at 96 HAT.

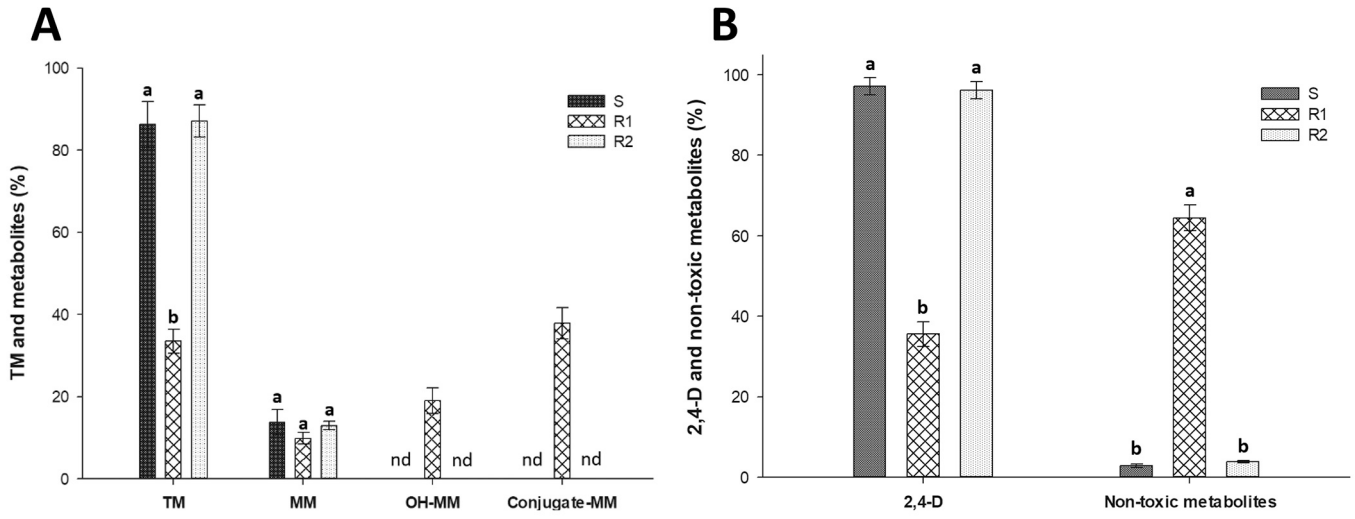


**Fig. 3.** Herbicide metabolites in S, R1 and R2 *P. rhoeas* populations at 96 HAT. A) TM and its metabolites (expressed as %) in plants treated with 20 g ai ha$^{-1}$; B) 2,4-D and its non-toxic metabolites (expressed as %) in plants treated with 400 g ai ha$^{-1}$.

**Table 4**
Number of *Papaver rhoeas* individuals with different amino acid changes at position 197. Position 574 is not shown since no mutations were found in all populations.

| Population | Pro/Pro | Thr/Ser | Phe/Ser | Phe/Leu | Phe/Thr | Ser/Ser | Ser/Pro |
|---|---|---|---|---|---|---|---|
| **S** | 12 | – | – | – | – | – | – |
| **R1** | – | 2 | 3 | 2 | 1 | 1 | 3 |
| **R2** | 12 | – | – | – | – | – | – |

acid position 197 for each of the 12 plants sequenced per population are shown Supplementary table 1.

### 3.6. ALS enzyme modelling and ligand docking

To better understand the impact of the mutations found in our resistant populations at the molecular level, we modeled the protein structure of *P. rhoeas* ALS and analyzed herbicide binding by in silico docking. The PrALS amino acid sequence was used as template for protein modelling (structure homology-modelling server, SWISS-MODEL, https://swissmodel.expasy.org/). The PrALS model generated was subjected to a structural assessment that was satisfactory with 95.51% of Ramachandran Favored Residues and a global quality QMEANDisCo score of 0.87 (Supporting Information: Fig. S2 A and B). Structural alignment of the *P. rhoeas* ALS model with the published structure of AtALS (AtALS, PDB:3E9Y) also revealed extensive overlap

between both structures, importantly at the catalytic core (Supporting Information: Fig. S2 C and D). We then used our modeled PrALS structure coordinates to simulate the binding of PrALS to the sulfonylurea TM, and to the imidazolinone (IMI), imazamox, using in-silico docking. Inspection of the binding mode of both herbicides into PrALS shows the conservation of the binding mechanism among plant ALS enzymes (Garcia et al., 2017; Lonhienne et al., 2018). As described for other plant species, TM and imazamox establish contacts with the residues Gly121 and Arg377 (Garcia et al., 2017) of the ALS tunnel. TM binding pose also shows that this SU is bound through a conserved mechanism and it is in much closer contact than imazamox with amino acids of the active site tunnel, including a pi-stacking interaction between the aromatic ring of TM and Trp574 of PrALS, missing in the case of imazamox (Fig. 4, Supporting Information: Fig. S3). However, the amino acid change of Pro197 for the bulkier Phe found in the resistant plants, provokes a reduction on the accessibility of the herbicide to the enzyme active site (Fig. 4D). Phe197 occupies a larger space in the entrance of the tunnel, preventing herbicide binding and conferring broad herbicide resistance (Fig. 4D). In the case of the Pro197Ser amino acid change found also in our resistant populations, the molecular mechanism for herbicide resistance seems different. The modeled PrALS$^{Pro197Ser}$ change leads to a local structural rearrangement that moves away several residues important for TM binding (Fig. 4D, Supporting Information: Fig. S3). Interestingly, this mutation is predicted to have no negative impact on imazamox binding because the interaction network of IMI herbicides is different from SU (Fig. 4C, Supporting Information: Fig. S3). According to docking results, imazamox is able to adopt a productive binding pose even in PrALS$^{Pro197Ser}$ protein (Fig. 4D, Supporting Information: Fig. S3).

### 3.7. Cross-resistance and alternative herbicides

According to the trials carried out in greenhouses with different herbicides on the control of the three populations of *P. rhoeas* (Table 5), the S population was totally controlled by all the tested herbicides (ALS inhibitor, auxin mimics, EPSPS inhibitor, PS I inhibitor and HPPD inhibitor), except for clopyralid (auxin mimic, pyridine-carboxylates chemical family) and pyraflufen (PPO inhibitor). For these two herbicides, the S population showed a higher natural tolerance (Table 5). The R1 population showed multiple resistance to ALS inhibitors and auxin mimics and cross-resistance to several chemical family herbicides from these two SoAs (TM, bispiribac-sodium, florasulam, flucarbazone, imazamox and 2,4-D, dicamba, fluroxypyr, MCPA), and natural tolerance to clopyralid and pyraflufen too (Table 5). The R2 population only showed cross-resistance to the auxin mimics, natural tolerance to clopyralid and pyraflufen, and was susceptible to all ALS-inhibiting herbicides tested. Both R1 and R2 populations were fully controlled by the applied EPSPS, PS I and HPPD inhibiting herbicides.

**Table 5**
Effect of alternative herbicides at field doses application on survival (%) of *Papaver rhoeas* plants from S (susceptible), R1 and R2 populations.

| Herbicide | S | R1 | R2 |
|---|---|---|---|
| **Control** | 100 | 100 | 100 |
| **Bispyribac-sodium**[a] | 0 | 100 | 0 |
| **Florasulam** | 0 | 100 | 0 |
| **Flucarbazone** | 0 | 100 | 0 |
| **Imazamox** | 0 | 100 | 0 |
| **Clopyralid** | 100 | 100 | 100 |
| **Dicamba** | 0 | 100 | 100 |
| **Fluroxypyr** | 0 | 100 | 100 |
| **MCPA** | 0 | 100 | 100 |
| **Pyraflufen** | 60 | 70 | 60 |
| **Glyphosate** | 0 | 0 | 0 |
| **Metribuzine** | 0 | 0 | 0 |
| **Mesotrione** | 0 | 0 | 0 |

[a] +BioPower (0.6 L ha$^{-1}$).

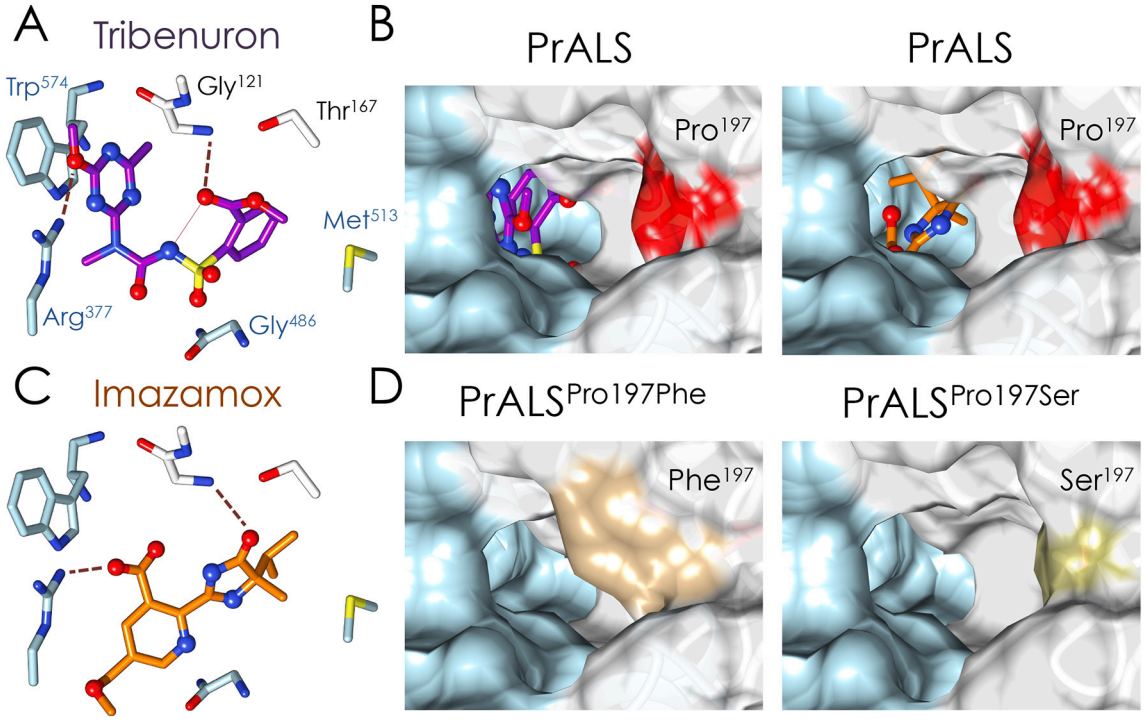


**Fig. 4.** Molecular docking of tribenuron and imazamox herbicides into *Papaver rhoeas* ALS enzyme (PrALS) structural models. (A) tribenuron and (C) imazamox binding mode in PrALS. Interactions amino acid-ligand are depicted with dashed red lines and intra-ligand interactions are shown in straight red lines. (B) and (D) Detail of the predicted tunnel structures for PrALS, PrALS$^{Pro197Phe}$ and PrALS$^{Pro197Ser}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

Herbicide resistance in *P. rhoeas* from Portugal is still pending full confirmation, though resistance to ALS inhibitors in populations from cereals has recently been reported (Torra et al., 2022). Since wheat monoculture is practiced in some regions, and ALS and auxin mimics are becoming one of the most used herbicides. Therefore, there is a high risk of selecting multiple R populations to these two SoAs. This study is reporting for the first time and confirming the presence of *P. rhoeas* populations from Portugal with multiple resistance to auxin mimics and ALS inhibitors. Multiple R populations to these SoAs are already reported in Spain, France, Italy and Greece (Heap, 2022). Also, several resistance mechanisms are reported for both SoAs: TSR (mutation) and NTSR (enhanced metabolism) for ALs inhibitors, and two NTSR mechanisms (enhanced metabolism and reduced transport) for auxin mimics. This variety of resistant mechanisms shows the genetic variability of the species and its capacity to accumulate both TSR and NTSR mechanisms within a population. The population R1 was characterized as multiple R to 2,4-D and TM, while R2 population was only 2,4-D R.

Regarding TSR mechanisms, point mutations corresponding to the codon Pro197 caused four different amino acid substitutions (Phe197, Leu197, Ser197 and Thr197). Changes in this position, particularly the Pro197Ser substitution, are reported in several European countries as conferring high levels of resistance to TM in *P. rhoeas* (Délye et al., 2011), (Rey-Caballero et al., 2017b), Kaloumenos et al., 2009, Marshall et al., 2010, Kati et al., 2019). ALS activity results further support the role of the detected mutations in the resistance to TM in R1 population. Regarding NTSR mechanisms, enhanced levels of P450-mediated metabolism were found in this population. Results from dose-response experiments using the P450 inhibitor malathion together with TM metabolism studies, support that R1 population is able to degrade TM in a three-steps metabolic route to MM, then to OH-MM, and finally to a sugar conjugate-MM, probably involving P450 in the first two steps. Previous studies with Spanish populations have reported P450-mediated enhanced metabolism of IMI (Torra et al., 2021a). Other ALS-inhibitors R broadleaved weed species can also metabolize TM (Hatami et al., 2016).

Another novelty regarding the resistance mechanisms to ALS inhibitors is the new mutation found for *P. rhoeas*, causing the amino acid substitution Pro197Phe. This is one of the few world cases reporting this mutation (Long et al., 2019; Murphy and Tranel, 2019). The Pro197Phe is the result of a double nucleotide substitution when compared to the wild-type sequence (Supplementary table 1), which might explain its rarity in field populations. This study represents the first attempt to undertake molecular docking with the PrALS enzyme. The Pro197Ser mutation would disrupt the important interactions between PrALS and herbicides in a different way compared to Pro197Phe. The Pro197Ser mutation would produce a structural rearrangement affecting the tunnel to the active site, moving away important amino acids for TM binding. The TM aromatic ring will adopt a non-productive pose and could not properly bind, because the number of hydrogen bonds would be reduced (Supporting Information: Fig. S3), as found in other broadleaved weeds (Yang et al., 2018). Docking analysis shows that while the Pro197Ser mutation prevents TM binding, the IMI imazamox can adopt a good pose and still binds to Pro197Ser (Supporting Information: Fig. S3), as previously found in *P. rhoeas* (Durán-Prado et al., 2004; Rey-Caballero et al., 2017b). On the contrary, the Pro197Phe mutation prevents the binding of both SU and IMI herbicides by a different mechanism. In this case, the bulker Phe substitution would obstruct the tunnel active site and most ALS inhibiting chemistries could not properly bind, conferring cross-resistance to most of them (Fig. 4). All plants treated with IMI, TP, PTB, and TZ at recommended label rates survived (Table 5), supporting the hypothesis that this new mutation for *P. rhoeas* could confer cross-resistance to all ALS-inhibiting chemistries and validating our docking predictions.

According to this research, resistance to 2,4-D can apparently be conferred by more than one mechanism in *P. rhoeas* from Portugal, by two distinct NTSR mechanisms: reduced transport and enhanced metabolism. Up to now, always both mechanisms were observed in previous studies on auxin mimic R populations from Spain, without establishing if they were related or independent mechanisms (Busi et al., 2018; Rey-Caballero et al., 2016; Torra et al., 2017). Since one population (R2) showed 2,4-D reduced translocation but not either metabolism or malathion synergism, it could be hypothesized that reduced translocation and P450-mediated metabolism could evolve as independent resistance mechanisms. It is interesting that RF to 2,4-D in R2 population were two to five times lower than those for the multiple R population R1 (Table 3), in which both P450-mediated enhanced metabolism together with reduced transport contribute to higher resistance levels. Finally, R1 and R2 populations were able to survive labeled rates of different auxin mimics chemistries (Table 5), so the two NTSR mechanisms may confer cross-resistance within this SoA. Further studies would be required to corroborate these hypotheses and unravel the genes involved in NTSR to auxin mimics in *P. rhoeas*.

Herbicide R *P. rhoeas* can accumulate several resistance mechanisms to ALS inhibitors and auxin mimics within a single population, both TSR and NTSR. For each SoA, it would be important to establish the prevalence and contribution of each mechanism, namely point mutations and P450-enhanced metabolism for ALS inhibitors, and reduced transport and also metabolism for auxin mimics. It has been reported that a single P450 enzyme could be able to metabolize imazamox and 2,4-D in this species (Torra et al., 2021a). Future studies should try to identify putative P450 genes encoding enzymes that can degrade 2,4-D, imazamox or TM, and establish if a single one or several can confer cross- and multiple resistance to ALS inhibitors and auxin mimics. Though some alternative herbicide SoAs to be included in an Integrated Weed Management (IWM) program are still effective in controlling herbicide R *P. rhoeas* (Table 5), a lot of caution must be taken. First, because the variety of resistance mechanisms detected in this research supports the high genetic variability and adaptability of this species (Torra and Recasens, 2008). Second, because P450-mediated enhanced metabolism can evolve in *P. rhoeas*, and its management based only on chemical management is at risk.

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## 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.2022.105226.

## References


Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. J. Appl. Toxicol. 34, 458–479.

Bond, W., Grundy, A.C., 2001. Non-chemical weed management in organic farming systems. Weed Res. 41, 383–405.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 7 (72), 248–254.

Busi, R., Goggin, D.E., Heap, I.M., Horak, M.J., Jugulam, M., Masters, R.A., Napier, R.M., Riar, D.S., Satchivi, N.M., Torra, J., et al., 2018. Weed resistance to synthetic auxin herbicides. Pest Manag. Sci. 74, 2265–2276.

Cirujeda, A., Recasens, J., Taberner, A., 2001. A qualitative quick-test for detection of herbicide resistance to tribenuron-methyl in *Papaver rhoeas*. Weed Res. 41, 523–534.

Cirujeda, A., Recasens, J., Torra, J., Taberner, A., 2008. A germination study of herbicide-resistant field poppies in spain. Agron. Sustain. Dev. 28 (2), 207–220.

Cruz-Hipolito, H., Rosario, J., Ioli, G., Osuna, M., Smeda, R., González-Torralva, F., De Prado, R., 2013. Resistance Mechanism to Tribenuron-Methyl in White Mustard (*Sinapis alba*) from Southern Spain. Weed Sci. 61 (3), 341–347.

Délye, C., Pernin, F., Scarabel, L., 2011. Evolution and diversity of the mechanisms endowing resistance to herbicides inhibiting acetolactate-synthase (ALS) in corn poppy (*Papaver rhoeas* L.). Plant Sci. 180, 333–342.

Délye, C., Jasieniuk, M., Le Corre, V., 2013. Deciphering the evolution of herbicide resistance in weeds. Trends Genet. 29, 649–658.

Durán-Prado, M., Osuna, M.D., De Prado, R., Franco, A.R., 2004. Molecular basis of resistance to sulfonylureas in *Papaver rhoeas*. Pestic. Biochem. Physiol. 42, 110–118.

Figueiredo, M.R.A., Küpper, A., Malone, J.M., Petrovic, T., de Figueiredo, A.B.T.B., Campagnola, G., Gaines, T.A., 2022. An in-frame deletion mutation in the degron tail of auxin coreceptor IAA2 confers resistance to the herbicide 2,4-D in *sisymbrium orientale*. PNAS USA 119 (9), e2105819119.

Garcia, M.D., Nouwens, A., Lonhienne, T.G., Guddat, L.W., 2017. Comprehensive understanding of acetohydroxyacid synthase inhibition by different herbicide families. PNAS USA 114 (7), E1091–E1100.

Hada, Z., Menchari, Y., Rojano-Delgado, A.M., Torra, J., Menéndez, J., Palma-Bautista, C., Souissi, T., 2021. Point mutations as main resistance mechanism together with P450-based metabolism confer broad resistance to different ALS-inhibiting herbicides in glebionis coronaria from tunisia. Front. Plant Sci. 12, 626702.

Hatami, Z.M., Gherekhloo, J., Rojano-Delgado, A.M., Osuna, M.D., Alcántara, R., Fernández, P., Sadeghipour, H.R., De Prado, R., 2016. Multiple mechanisms increase levels of resistance in *Rapistrum rugosum* to ALS herbicides. Front. Plant Sci. 7, 169.

Heap, I., 2022. International Survey of Herbicide Resistant Weeds. Annual Report Internet. Available at: http://www.weedscience.com (accessed 13th June 2022).

Holm, L., Doll, J., Holm, E., Pancho, J., Herberger, J., 1997. World Weeds: Natural Histories and Distribution. John Wiley, New York, NY, USA, p. 129.

Kaloumenos, N.S., Dordas, C.A., Diamantidis, G.C., Eleftherohorinos, I.G., 2009. Multiple Pro197 substitutions in the acetolactate synthase of corn poppy (*Papaver rhoeas*) confer resistance to tribenuron. Weed Sci. 57, 362–368.

Kati, V., Scarabel, L., Teiery-Lanfranchi, D., Kioleoglou, V., Liberopoulou, S., Delye, C., 2019. Multiple resistance of *Papaver rhoeas* L. to 2,4-D and acetolactate synthase inhibitors in four European countries. Weed Res. 59, 367–376.

LeClere, S., Wu, C., Westra, P., Sammons, R.D., 2018. Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *kochia scoparia* is endowed by a mutation in an AUX/IAA gene. PNAS USA 115 (13), E2911–E2920.

Long, W., Malone, J., Boutsalis, P., Preston, C., 2019. Diversity and extent of mutations endowing resistance to the acetolactate synthase (AHAS)-inhibiting herbicides in Indian hedge mustard (*Sisymbrium orientale*) populations in Australia. Pestic. Biochem. Physiol. 157, 53–59.

Lonhienne, T., Garcia, M.D., Pierens, G., Mobli, M., Nouwens, A., Guddat, L.W., 2018. Structural insights into the mechanism of inhibition of AHAS by herbicides. PNAS USA 115 (9), E1945–E1954.

Lutman, J.W., Cussans, G.W., Wright, K.J., Wilson, B.J., McN Wright, G., Lawson, H.M., 2002. The persistence of seeds of 16 weed species over six years in two arable fields. Weed Res. 42, 231–241.

Marshall, R., Hull, R., Moss, S.R., 2010. Target site resistance to ALS inhibiting herbicides in Papaver rhoeas and *Stellaria media* biotypes from the UK. Weed Res. 50, 621–630.

Mithila, J., Hall, J.C., Johnson, W.G., Kelley, K.B., Riechers, D.E., 2011. Evolution of resistance to auxinic herbicides: historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Sci. 59, 445–457.

Mitich, L.W., 2000. Corn poppy (*Papaver rhoeas* L.). Weed Technol. 14, 826–829.

Mora, A.D., Rosario, J., Rojano-Delgado, A.M., Palma-Bautista, C., Torra, J., Alcántara-De La Cruz, R., De Prado, R., 2019. Multiple resistance to synthetic auxin herbicides and glyphosate in *parthenium hysterophorus* occurring in citrus orchards. J. Agric. Food Chem. 67 (36), 10010–10017.

Moss, S.R., 2004. Herbicide-resistant weeds in Europe: the wider implications. Commun. Agric. Appl. Biol. Sci. 69, 3–11.

Murphy, B.P., Tranel, P.J., 2019. Target-site mutations conferring herbicide resistance. Plants 8, 382.

Oerke, E.-C., 2006. Crop losses to pests. J. Agric. Sci. 144, 31–43.

Palma-Bautista, C., Belluccini, P., Gentiletti, V., Vázquez-García, J.G., Cruz-Hipolito, H. E., De Prado, R., 2020. Multiple Resistance to Glyphosate and 2,4-D in *Carduus acanthoides* L. from Argentina and Alternative Control Solutions. Agron. 10, 1735.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera–a visualization system for exploratory research and analysis. J. Comput. Chem. 25 (13), 1605–1612.

Powles, S.B., Yu, Q., 2010. Evolution in action: plants resistant to herbicides. Annu. Rev. Plant Biol. 61, 317–347.

Rey-Caballero, J., Menéndez, J., Giné-Bordonaba, J., Salas, M., Alcántara, R., Torra, J., 2016. Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Pestic. Biochem. Physiol. 133, 67–72.

Rey-Caballero, J., Royo-Esnal, A., Recasens, J., González, I., Torra, J., 2017a. Management options for multiple herbicide-resistant corn poppy (*papaver rhoeas*) in spain. Weed Sci. 65 (2), 295–304.

Rey-Caballero, J., Menedez, J., Osuna, M.D., Salas, M., Torra, J., 2017b. Target-site and non-target-site resistance mechanisms to ALS inhibiting herbicides in *Papaver rhoeas*. Pestic. Biochem. Physiol. 138, 57–65.

Rosario, J.M., Cruz-Hipolito, H.E., Smeda, R.J., De Prado, R., 2011. White mustard (*Sinapis alba*) resistance to ALS-inhibiting herbicides and alternative herbicides for control in Spain. Eur. J. Agron. 10, 101–110.

Scarabel, L., Pernin, F., Delye, C., 2015. Occurrence, genetic control and evolution of non-target-site based resistance to herbicides inhibiting acetolactate synthase (ALS) in the dicot weed *Papaver rhoeas*. Plant Sci. 238, 158–169.

Shaner, D.L., 2014. Herbicide handbook, 10th ed. Weed Science Society of America, Lawrence, KS.

Song, Y., 2014. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. J. Integr. Plant Biol. 56, 106–113.

Todd, O.E., Figueiredo, M.R.A., Morran, S., Sonia, N., Preston, C., Kubeš, M.F., Napier, R., Gaines, T.A., 2020. Synthetic auxin herbicides: finding the lock and key to weed resistance. Plant Sci. 300, 110631.

Torra, J., Recasens, J., 2008. Demography of corn poppy (*Papaver rhoeas*) in relation to emergence time and crop competition. Weed Sci. 56, 826–833.

Torra, J., Cirujeda, A., Recasens, J., Taberner, A., Powles, S.B., 2010. PIM (poppy integrated management): a bio-economic decision support model for the management of *papaver rhoeas* in rain-fed cropping systems. Weed Res. 50 (2), 127–139.

Torra, J., Rojano-Delgado, A.M., Rey-Caballero, J., Royo-Esnal, A., Salas, M.L., Prado, D., R., 2017. Enhanced 2,4- D metabolism in two resistant *Papaver rhoeas* populations from Spain. Front. Plant Sci. 8, 1584.

Torra, J., Rojano-Delgado, A.M., Menéndez, J., Salas, M., De Prado, R., 2021a. Cytochrome P450 metabolism-based herbicide resistance to imazamox and 2,4-D in *Papaver rhoeas*. Plant Physiol. Biochem. 160, 51–56.

Torra, J., Osuna, M.D., Merotto, A., Vila-Aiub, M., 2021b. Editorial: Multiple herbicide-resistant weeds and non-target site resistance mechanisms: a global challenge for food production. Front. Plant Sci. 12, 763212.

Torra, J., Montull, J.M., Calha, I.M., Osuna, M.D., Portugal, J., de Prado, R., 2022. Current status of herbicide resistance in the Iberian Peninsula: future trends and challenges. Agron. 12, 929.

Tranel, P.J., Wright, T.R., 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci. 50, 700–712.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46, W296–W303.

Wilson, B.J., Wright, K.J., Brain, P., Clements, M., Stephens, E., 1995. Predicting the competitive effects of weed and crop density on weed biomass, weed production and crop yield in wheat. Weed Res. 35, 265–278.

Yang, Q., Deng, W., Wang, S., Liu, H., Li, X., Zheng, M., 2018. Effects of resistance mutations of Pro197, Asp376 and Trp574 on the characteristics of acetohydroxyacid synthase (AHAS) isozymes. Pest Manag. Sci. 74, 1870–1879.