Target-site and Non-target-site resistance mechanisms to ALS inhibiting herbicides in *Papaver rhoeas*

Jordi Rey-Caballero[a], Julio Menéndez[b], Maria D. Osuna[c], Marisa Salas[d], Joel Torra[a*]

* Correspondence to: Joel Torra Farré, Department D'Hortofructicultura, Botànica i Jardineria, Agrotecnio, Universitat de Lleida, Alcalde Rovira Roure 191, Lleida, Spain. E-mail: joel@hbj.udl.cat

[a]Department d'Hortofructicultura, Botànica i Jardineria, Agrotecnio, Universitat de Lleida, Alcalde Rovira Roure 191, Lleida, Spain

[b]Departamento de Ciencias Agroforestales, Escuela Politécnica Superior, Campus Universitario de La Rábida, 21071 Palos de la Frontera, Huelva, Spain.

[c]"Finca La Orden-Valdesequera" Research Centre, Ctra. A-V, Km372, 06187 Guadajira Badajoz, Spain.

[d] DuPont de Nemours, Reu Delarivière Lefoullon, La Defense Cedex, Paris 92064, France

Abstract

Target-site and non-target-site resistance mechanisms to ALS inhibitors were investigated in multiple resistant (tribenuron-methyl and 2,4-D) and only 2,4-D resistant, Spanish corn poppy populations. Six amino-acid replacements at the Pro197 position (Ala197, Arg197, His197, Leu197, Thr197 and Ser197) were found in three multiple resistant populations. These replacements were responsible for the high tribenuron-methyl resistance response, and some of them, especially Thr197 and Ser197, elucidated the cross-resistant pattern for imazamox and florasulam, respectively. Mutations outside of the conserved regions of the ALS gene (Gly427 and Leu648) were identified, but not related to resistance response. Higher mobility of labeled tribenuron-methyl in plants with multiple resistance was, however, similar to plants with only 2,4-D resistance, indicating the presence of non-target-site resistance mechanisms (NTSR). Metabolism studies confirmed the presence of a hydroxy imazamox metabolite in one of the populations. Lack of correlation between phenotype and genotype in plants treated with florasulam or imazamox, non-mutated plants surviving imazamox, tribenuron-methyl translocation patterns and the presence of enhanced metabolism revealed signs of the presence of NTSR mechanisms to ALS inhibitors in this species. On this basis, selection pressure with ALS non-SU inhibitors bears the risk of promoting the evolution of NTSR mechanisms in corn poppy.

Keywords: enhanced metabolism, genotype, mutation, phenotype, synthetic auxins, translocation pattern.

1. Introduction

Acetohydroxy acid synthase (AHAS, EC 4.1.3.18), also referred to as acetolactate synthase (ALS, EC2.2.1.6), is the first enzyme involved in the biosynthesis of branched chain amino-acids valine, leucine and isoleucine [1,2]. This enzyme is the target site of five herbicide chemical groups: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-thiobenzoates (PTB) and sulfonyl-aminocarbonyl-triazolinones (SCT). These herbicides, commonly referred to as ALS inhibiting herbicides, are highly effective at a low rate and environmentally safe [2]. Only five years after the introduction of the first SU, resistant biotypes *Lactuca serriola* L. [3] and *Kochia scoparia* L. [4] were reported. To date, 155 species in locations all over the world (94 dicots and 61 monocots) have evolved resistance to ALS inhibitors [5].

The SU and IMI herbicides are not competitive inhibitors of ALS because they do not directly bind to the substrate's active site. Instead, these herbicides bind within the substrate-access channel of the ALS enzyme in plants. In this way, both herbicides inhibit ALS by blocking substrate access to the active site. It is well documented that SU are better ALS inhibitors than IMI because SU fit better (more hydrogen bonds are involved) and deeper into the channel (closer to the active site) [2]. In most cases, resistance to ALS inhibitors is caused by mutation of the ALS gene, which results in the change of a single amino-acid residue in the herbicide-binding site (Target-site resistance, TSR) [6]. Thus far, 28 amino-acid substitutions endowing ALS inhibitors resistance have been reported, mainly at the Pro197 site (Ala, Arg, Asn, Gln, His, Ile, Leu, Lys, Met, Ser, Thr, Trp and Tyr), and also at Ala122 (Thr, Tyr and Val), Ala205 (Val), Asp376 (Glu), Trp574 (Arg, Leu, Gly and Met), Ser653 (Asn, Ile and Thr) and Gly654 (Glu and Asp) in resistant weed species [5,7]. There is a wide variation in the resistant response among species with a given substitution [7]. Moreover, ALS-inhibitors cross-resistance is also dependent on specific mutations, chemical groups, specific herbicides within a given group, and sometimes even weed species [8]. Generally, a high level of resistance is conferred by Pro197 substitutions to SU and by Trp574 substitutions to all classes of ALS inhibitors. A second mechanism of resistance to ALS inhibitors is to reduce the amount of herbicide reaching ALS to be below the lethal level (Non-target-site resistance, NTSR). Reduced absorption and translocation rarely underlay resistance to ALS inhibitors [9-12], and in only a few cases have they been reported as a partial resistance mechanism [13, 14]. On the other hand, an enhanced herbicide metabolism rate has been proposed for several species, such as *Lolium rigidum* L. [15], *Sinapis arvensis* L. [9] and *Echinochola phyllopogon* L. [16].

An amalgam of different factors has been proposed to contribute to the number of ALS inhibitor-resistant cases. Additionally, the repeated use of these herbicides is an most important aspect [6], though genetic, molecular and physiological biology of this resistance must also be considered. High mutation rates in ALS genes of some species account for the relatively high frequency of resistant alleles to ALS inhibitors in natural populations [17, 18]. Moreover, resistant ALS alleles are dominant over susceptible alleles and because ALS is a nuclear gene, resistant alleles are disseminated by both pollen and seed [6]. Studied resistant species have not shown any fitness cost associated to the most common mutations of the ALS gene (Pro197 and Trp574) [19-21]. For this reason, it has been considered that these resistant characteristics will persist in the populations and not decline with the time [8].

Papaver rhoeas L. (corn poppy) is the most common dicotyledonous weed in winter cereals in southern Europe [22]; it is an annual, diploid species that is insect-pollinated and self-incompatible [23]. In recent years, corn poppy with multiple resistance to 2,4-D and tribenuron-methyl has been reported in Spain [24] and Italy [20], and independent resistance to ALS inhibitors has evolved in a number of other countries across Europe (Belgium, Denmark, France, Germany, Greece, Poland, Sweden and United Kingdom) [5]. In Spain, the resistance to tribenuron-methyl is conferred by Pro197 to Ser substitutions [25]. In addition, irregular responses to other ALS inhibitors (mainly IMI and TP) have been reported in post-emergence field applications. Recently, the presence of NTSR mechanisms in Italian corn poppy has been shown because plants resistant to imazamox, but not carrying mutant ALS alleles, were identified [26]. These resistance mechanisms, genes involved and how they affect the different ALS inhibitor chemistries still needs to be uncovered.

The objectives of this study were (1) determine, with dose-response experiments, the herbicide rate causing 50% mortality (GR_{50}) and the resistance index (RI) of resistant (R) and a susceptible (S) populations, primarily to ALS inhibitors (tribenuron-methyl, florasulam and imazamox), and secondarily to 2,4-D; (2) sequence the ALS gene from these corn poppy populations in order to identify potential mutations; (3) compare the genotype with the phenotype of individual plants in order to establish a relationship between the molecular results and the ALS inhibitors response; (4) study tribenuron-methyl absorption and translocation patterns; and (5) determine the presence of enhanced metabolism to imazamox to unveil potential NTSR mechanisms contributing to the resistance response of these corn poppy populations.

2. Materials and methods

2.1 Plant material

Before winter cereal harvest, mature corn poppy capsules were collected from four fields where corn poppy control with ALS inhibitors and/or 2,4-D had been reported as a failure. In addition, seeds from two susceptible (S) populations were obtained; one was provided by a seed dealer (Herbiseed, Twyford, UK) and the other was collected from the same region where suspicious resistant populations were collected. Further details regarding these populations are summarized in Table 1. Corn poppy seeds previously sterilized in a 30% hypochlorite solution were sown in Petri dishes with 1.4% agar supplemented with 0.2% KNO_3 and 0.02% gibberellin GA_3. Seeds were placed in a growth chamber at 20/10 °C day/night, a 16 h photoperiod under 350 µmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in 7 × 7 × 7 cm plastic pots filled with a silty loam:sand:peat (40% w/v, 20% w/v, 40% w/v) potting mix. Pots were placed in a greenhouse in Lleida, Spain (41°37’43.1”N -

0°35’52.6”E) and were watered and fertilized as needed. All plants produced in this manner were employed in the subsequent experiments.

2.2 Dose-response assays

Five seedlings were sown per pot and after sprouting, they were thinned to three per pot. At the six leaf stage (a 5-6 cm rosette), plants were sprayed with tribenuron-methyl, florasulam, imazamox or 2,4-D at a range of herbicide rates (rates are detailed in Table 2). Four replicates (pots) were applied with each herbicide rate. Herbicides were applied using a precision bench sprayer with two Hardi ISO LD-110-02 flat fan 110º opening nozzles operating at a forward speed of 0.9 ms^{-1}, 50 cm above plants, 200 l ha^{-1}, and at a pressure of 215 kPa. Four weeks after treatment, plants were harvested (above ground). Samples were dried at 65 °C for 48h, and the dry weights were measured. Finally, weight reduction was calculated as a percentage of the untreated control for each population. Experiments were repeated twice.

2.3 DNA extraction, ALS gene sequencing and restriction analysis

In another experiment, at the six leaf stage, a total of fifty-one plants per population (255 overall) were sprayed with tribenuron-methyl, florasulam or imazamox (seventeen plants for each product) at the recommended field rate. Plants from the S-012 population were not included in this experiment, but results from unpublished work did not detect any mutation among thirty plants. The herbicide was applied as described above. One week before application, a leaf fragment (~100 mg) from each plant was taken and frozen for subsequent molecular analyses. Four weeks after treatment, individual plant responses were evaluated. Dead plants were classified as susceptible (S). Plants re-growing from the center of the rosette were classified as moderately resistant (r) and plants that were unaffected by herbicide were classified as resistant (R) (Fig. 1). DNA from the leaf fragment was extracted using the Speedtools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Valle de Tobalina, Madrid, Spain) and the DNA sample concentration was measured in a NANODROP Thermoscientific spectrophotometer (ThermoFisher, Nano- Drop Products, Wilmington, DE). Each DNA sample was diluted to a final concentration of 10 ng/µl, which was immediately used for the polymerase chain reaction (PCR) test or stored at -20ºC until use.

Mutations conferring ALS resistance in corn poppy at Pro197 and Trp574 codons were analysed first for all the samples. Fragments of the ALS gene that included the regions of those codons were amplified using corn poppy primers described in a previous work [27]. The amplification was accomplished following the procedures described in the above mentioned work [27]. PCR amplification products were separated in a 1.5% agarose gel. Gels were then observed under ultraviolet light (320 nm; ALPHA DIGI DOC Pro instrument, Alpha Innotec Corporation, Johannesburg, South Africa) and images recorded with gel photography. Amplified DNA fragments were purified using the Speed tools PCR Clean-Up Kit (Biotools, B&M Labs, Madrid, Spain), then sequenced. Restriction analysis were conducted to define double-peaks detected in the sequence chromatograms. For this analysis, primers and procedures were utilized as described by Kaloumenos *et al.* [27]. The resulting electrophoresis bands were visualized under UV light after being stained with GelRed (Biptium, California, USA). The digestion profile for each population was compared with its respective, non-digested control profile as well as the S-control digestion profile. Haplotype inference was determined by comparing sequences obtained from the other samples within the same population. In some specific cases where the same genotype at Pro197 codon expressed different responses to the same herbicide, other positions related to ALS resistance were examined (Ala122 ,Pro197, Ala205, Asp376, Trp574, Ala653 and Gly654). Methodology for this part was conducted as described in a previous work [23].

2.4 Tribenuron-methyl absorption and translocation experiment

[^{14}C]-tribenuron-methyl ([^{14}C]-Tri) with a specific activity of 1.422 MBq/mmol (Institute of Isotopes Co. Ltd. Budapest, Hungary) was mixed with commercial formulated tribenuron-methyl in distilled water up to a final concentration of 0.093 g L^{-1} (18.7 g a.i.·ha^{-1} dissolved into 200 L ha^{-1} of distilled water). Four 0.5 μL droplets of this mixture were applied per plant to the adaxial surface of the fourth leaf at the six true leaf stage of development (a 5-6 cm rosette). Every plant received a radioactivity of 166.5 MBq $mmol^{-1}$. Five repetition (considering every plant as a repetition) from each population were harvested at 12, 24, 48, and 96 h after treatment (HAT). Unabsorbed [^{14}C]-Tri was rinsed from the treated leaves of each plant using 2 ml of an acetone and water (1:1 v/v) solution. The rinse of each replication was mixed with 15 mL scintillation fluid (UltimaGold^{TM}, Perkin-Elmer, Packard Bioscience BV), and analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA scintillation counter; Beckman Instruments, CA, USA). Washed plants were separated into treated leaf, shoots and root, dried at 70ºC for 48 h and parts were combusted in a sample oxidizer (OX 500; R. J. Harvey Instrument, Tappan, NY, USA). The radioactivity of the resulting [^{14}C]-CO_2 was determined by LSS. Foliar absorption (%) was calculated as: (Radioactivity recovered from plant parts) ⁄ (Total radioactivity recovered) x 100. Translocation (%) was calculated as: (Absorbed radioactivity in treated leaf, shoot or root) ⁄ (Absorbed radioactivity in all tissues) x 100. Percentage or recovery was always greater than 80%.

To assess translocation of [14C]-Tri, two treated plants for S-013, R-703 and R-213 populations were removed from pots 96 HAT. Roots were rinsed and whole plants were dried (65 °C for 48 h) and pressed against a 25 by 12.5–cm phosphor storage film (PerkinElmer Life and Analytical Sciences, Shelton, CT) for 6 h, and scanned using a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV).

2.5 Imazamox metabolism studies

The methodology followed was described by Rojano-Delgado et al. [28]. Application of imazamox was performed as dose–response assays with a relative volume of 200 L Ha^{-1} and with a dose of imazamox at 100 g ai Ha^{-1} (corresponding to two times the average dose used in field) as well as a 0 dose (control). Plants treated with herbicide and the controls were cut 0, 24, 48 and 72 h after application and stored at −40 °C until use. The frozen samples were washed with 60 mL of water to remove traces of imazamox and soil on the leaf surface, before the extraction. They were ground to a fine powder using liquid nitrogen in a porcelain mortar. One-half gram of each sample was mixed with 10 mL in proportion 90:10 (v/v) methanol–water and ultrasonicate at 70 W during 10 min (duty cycle 0.7 s s^{-1}). Then it was centrifugated (15 min at 20,000 rpm) to separate the solid residue. 6 mL of this extract was taken and evaporated to dryness under an air stream and later, reconstituted by 0.5 mL of extractant (90:10 methanol–water) and filtered through a nylon filter siringe (45 µm pore size and 13 mm i.d. from Millipore, Carrigtwohill, Ireland) before chromatographic analysis.

Imazamox and metabolites were determined by the liquid chromatography–DAD (diode array detector analysis) at a measurement wavelength of 240 nm. A Gold HPLC (High-performance liquid chromatography) System from Beckman Coulter (Fullerton, USA) equipped with a 26 System Gold Diode Array detector (wavelength range 190–600 nm) was used in this case. A hydrophilic interaction liquid chromatography column (20 cm x 4.6 cm, 3 µm particle size) was used for the separation of the metabolites and herbicide. 50 µL of the reconstituted phase was injected into the liquid chromatography with 1 % (v/v) acetic acid in water as mobile phase A and pure methanol as mobile phase B. The elution program started with 5 % mobile phase B and followed the linear gradient: step 1, 5 to 20 % methanol in 10 min; step 2, 20 to 80 % methanol in 10 min; step 3, 80 to 100 % methanol in 5 min; and step 4, 100 to 5 % methanol in 10 min. The constant flow rate and column temperature were 1.0 mL min^{-1} and 40 °C, respectively. Chromatographic peaks in the liquid chromatography–diode array detector were assigned according to retention times using as a reference the imazamox peak identified by spiking extracts with the commercial standard. Quantification of imazamox metabolites was based on the calibration model for imazamox, and the results were expressed as micrograms (µg) of the analyte equivalent to imazamox per gram (g^{-1}) of plant. Liquid chromatography–DAD was performed by using two plants per sample (three repetitions) and four populations (S-013, R-213, R-313 and R-703). Finally, mutations conferring ALS resistance in corn poppy at Pro197 and Trp574 codons were analysed for plants used at 72 HAT, six per population, as described above.

2.6 Statistical analysis

For the dose-response experiment, statistical analysis were carried out with a nonlinear regression model with the *drc* [29] package in R [30]. The herbicide rate causing 50% of plant growth reduction (GR_{50}) was calculated via four parameter logistic curves:

$$y = \mathrm{c} + \frac{(d-c)}{1+\mathrm{EXP}[b(\log(x)-\log(GR50))]} \quad (1)$$

Where c = the lower limit, d = the upper limit and b = the slope at the GR_{50}. In this regression equation, the herbicide rate (g a.i.·ha^{-1}) was the independent variable (x) and the dry weight (percentage of the untreated control for each population) was the dependent variable (*y*). The resistance index (RI) was computed as GR_{50}(R)/GR_{50}(S). Analysis of variance (ANOVA) was conducted with [^{14}C]-Tri percentages and imazamox quantities. Data were transformed as needed (arcs[√(x+0.5)]) when normal assumptions were not met. Population means from each evaluation time were compared using a post-hoc Tukey´s pairwise test [30], at P = 0.05. Data were then back transformed for their presentation.

3. Results

3.1 Dose-response experiments

R-213, R-313 and R-114 plants were 286-, 695- and 351-fold more resistant to tribenuron-methyl than susceptible plants (Table 3, Fig. 2). The R-703 population displayed a very small resistant index (RI) to tribenuron-methyl Florasulam GR_{50} was 0.16 g a.i. ha^{-1} in S-013 plants; this parameter was increased 24-fold in R-213 plants (3.90 g a.i. ha^{-1}) and 18-fold in both R-313 and R-114 populations (2.90 and 2.92 g a.i. ha^{-1} respectively). R-703 plants were two times more resistant to florasulam than H-S013 plants The GR_{50} value for imazamox in the susceptible population (S-013) was 0.61 g a.i. ha^{-1}. This parameter was 30 (18.08 g a.i. ha^{-1}), 40 (24.37 g a.i. ha^{-1}) and 24 (14.73 g a.i.ha^{-1}) times greater in R-213, R-313 and R-114 populations, respectively. R-703 plant results exposed them to be 6 times more resistant (a GR_{50} value of 4.05 g a.i. ha^{-1}) to imazamox than the susceptible biotype. Dose-response experiments conducted with 2,4-D revealed that all populations were resistant to 2,4-D, their RI's ranging from 12 to 18 (Table 3, Fig. 2). Minimal differences in the responses of the two S population responses were observed for the different tested herbicides.

3.2 ALS sequencing

No substitutions at codon Pro197 were found in S-013 plants. However, six amino-acid replacements were identified at this position (Ala197, Arg197, His197, Leu197, Ser197 and Thr197) in populations R-213, R-313 and R-114. Only one plant out of fifty-one in population R-703 presented a substitution (Thr197). Six different genotypes were identified in R-213 (Table 4), with 76% of the plants being classified as homozygous mutant (RR), 24% heterozygous mutant (RS), and 0% wild type (SS). In R-313, twelve different genotypes were detected, 76%, 20% and 4% of these plants were RR, RS and SS, respectively. Finally, eight different genotypes were observed in R-114 plants, 61% of the plants belonging to this population were characterised as RR, 25% as RS and 14% as SS (Table 4).

Results obtained by the multiple resistant populations (R-213, R-313 and R-114), revealed that all plants carrying at least one mutant ALS allele showed a R response to tribenuron-methyl treatments. The majority of the plants assayed with imazamox were classified as R or r. Most of the imazamox R plants carried at least one Thr197 or Leu197 allele. Few plants treated with florasulam were R, most of them were r or S. The majority of the plants which did not survive florasulam application had a Thr197 substitution (Pro/Pro genotype not included), and all plants with R response to this herbicide carried at least one Ser197 allele (Table 4). No mutant plants were found in the S-013 population and no survivors were found for either the tribenuron-methyl or florasulam applications, nevertheless only one plant survived the imazamox application (r). All plants in the R-703 population died when they were sprayed with tribenuron-methyl or florasulam, however, five plants survived imazamox (r). Only one plant presented a substitution (Thr/Thr) at position 197 (Table 4).

Some plants identically genotyped (carrying the same number of copies of one given mutant ALS allele) at Pro197 codon displayed different responses to florasulam or imazamox (Table 5). Moderate imazamox resistance (r) and susceptible (S) responses from S-013 and R-703 populations did not show any difference at the other studied positions (Table 5). Samples 25 and 26 from the population R-114 were r and R (respectively) to imazamox and both plants carried a Leu/Thr substitution at position 197. Additionally, these plants displayed a heterozygous mutation at position 427 (Glu/Lys) and only sample 26also carried a heterozygous mutation at position 648 (Leu/Ser). Samples 20 and 17 from population R-114 showed a Thr homozygous mutation at position 197, but r (sample 20) and S (sample 17) responses to florasulam were observed. Apart from this, both plants also carried a homozygous mutation at position 427 (Lys/Lys) (Table 5).

3.3 [^{14}C]-tribenuron-methyl experiments

There were no differences in [^{14}C]-Tri absorption patterns between corn poppy populations (Fig. 3). In addition, there were not significant time-related differences in terms of [^{14}C]-Tri absorption among all the populations tested, with percentages ranging from 24.3% (at 12 HAT) to 37.8 % (at 96 HAT) of the recovered radioactivity (Fig. 3). There were differences in [^{14}C]-Tri translocation between populations starting 48 HAT, with a maximum at 96 HAT, where they were statistically significant (Fig. 3). While radioactivity in susceptible plants remained asymptotic, radioactivity evaluated in the treated leaf of R-213, R-313, R-114 and R-703 decreased. Therefore, at 96 HAT the percentage of [^{14}C]-Tri found in the treated leaf of the S plants was 70.9%, which was statistically different from the rates obtained for the rest of populations. The lowest amount of radioactivity in a treated leaf at 96 HAT was detected in R-313 plants (27.6% of the penetrated radioactivity), while [^{14}C]-Tri for R-213, R-114 and R-703 plants ranged from 45.4 to 49.3% (Fig. 3). These data were consistent with those observed in the shoot, where significant differences were only detected at 96 HAT. R-313 plants translocated almost 3-fold more [^{14}C]-Tri to the shoots (68.9%) than S-013 plants (25.6%). Radioactivity detected in R-213, R-114 and R-703 shoots at the same evaluation time was 46.8, 49.1 and 51.2%, respectively (Fig. 3). No differences between populations in terms of herbicide translocation to roots were detected at any evaluation time, thus radioactivity evaluated in this part was negligible (Fig. 3). Percentages of recovered radioactivity ranged from 80 to 88% in the S-013 population, from 85 to 99%, from 80 to 85%, from 77 to 97% and from 80 to 86% in R-213, R-313, R-114 and

R-703 populations, respectively (data not show). Images obtained from the qualitative studies at 96 HAT confirmed the above results (Fig. 4).

3.4 Imazamox metabolism studies

The imazamox metabolic pattern was different between the S and the R corn poppy populations at 48 and 72 HAT (Table 6). At both sampling times, significantly more herbicide was detected in the S population. At 72 HAT, the R-313 population had significantly the lowest amount of herbicide in the aerial part of the plants. Imazamox was only detected in roots in the S population at 48 HAT, while at 72 HAT the amount was significantly much higher compared to R ones. The liquid chromatography-DAD revealed the presence of a hydroxy metabolite of imazamox (at 17 min in the chromatogram, Supplementary Material) in the aerial part and roots in one of the R populations, R-313, at 72 HAT (Table 6). The amount of the metabolite was much higher in aboveground part of the plants. For plants sampled at 72 HAT, ALS gene sequence revealed only wild type genotypes at Pro197 codon in the susceptible (S-013) and the synthetic auxin resistant (R-703) populations. In multiple resistant populations, four Ser/Ser and two Pro/Ser mutants were found in R-313, and three Ser/Ser, one Pro/Ser and one Thr/Pro. None had an aminoacid change at Thr574 codon.

4. Discussion

Multiple resistance to ALS inhibiting herbicides and 2,4-D was detected in R-213, R-313 and R-114 corn poppy populations. GR_{50} values for these products were consistent with those reported in Greek ALS resistant and multiple resistant corn poppy populations [32]. As observed in previous studies [33], the degree of resistance varied among ALS inhibitors, resistant factors being much lower for florasulam and imazamox than for tribenuron-methyl.

In this study, six amino-acid replacements at the Pro197 position have been found (Ala197, Arg197, His197, Leu197, Thr197 and Ser197); the first five replacements being new for Spanish corn poppy populations, and consistent with previously published European works [23, 27, 34]. The strong resistance to tribenuron-methyl showed by any kind of substitution at Pro197 is because Pro197 amino-acid residue is directly involved in anchoring the aromatic ring of SU. Any replacement in this position will affect SU binding, resulting in strong resistance to this herbicide [2, 35]. Cross-resistance patterns between ALS inhibitors depends on both the codon mutated and the specific amino-acid replaced at the codon [36]. Due to this, different substitutions at Pro197 can give strong, moderate, or no resistance among IMI and TP. In concordance with results in another study [23], corn poppy plants carrying the Thr197 substitution were resistant or moderately resistant to imazamox. Although Pro197 is not involved in binding IMI [37], certain substitutions of these amino-acid residues may result in IMI resistance because the replacement of Pro by a bulky amino-acid obstructs the entry of IMI into the ALS tunnel [2]. Regarding TP, results of this work show that the substitution of Pro197 by Ser lead to plants that were moderately cross-resistant to florasulam, as observed by Délye *et al.* [23].

The overuse of tribenuron-methyl during the early 80's in Spanish fields, probably selected a wide variety of Pro197 substitutions in corn poppy. Consecutive ALS herbicide management practices in each field contributed to the reduction, or not, of ALS genotype diversity, depending on which ALS herbicide families were predominantly used. This case is clearly apparent in R-213 plants continuously treated with florasulam + 2,4-D in recent years, as this population has the highest florasulam resistant index, together with the highest Ser allele frequency reported.

Plants carrying a double mutation at positions Pro197 and Gly427 (by Lys), and a triple mutation at positions Pro197, Gly427 and Leu648 (by Ser), were detected in this study. Results from a previous work conducted with ALS resistant corn poppy from Spain also detected a point mutation located outside the conserved domains: a replacement of Gly281 by Glu [25]. However, plants carrying these mutations displayed different responses to the same herbicide. Therefore no implication of these two new mutations in resistance response was assumed, awaiting further confirmation.

In this research, plants with the same genotype at ALS did not always show the same phenotype when they were treated with florasulam or imazamox. Analogous results were reported in ALS inhibitors resistant *Raphanus raphanistrum* L. and *P. rhoeas* [26, 34]. Délye *et al.* [23] and Scarabel *et al.* [26] demonstrated that a NTSR to ALS inhibitors, yet to be determined, could be behind the mismatch between the genotype and phenotype. Moreover, five plants without any mutation were able to survive imazamox application among all populations. In other weeds, NTSR mechanisms (metabolism related) were assumed to be present by identifying sensitive ALS in plants with resistant phenotypes [8, 26, 38]..

As observed in corn poppy, no differences in absorption between resistant and susceptible biotypes were also reported in other studies conducted with ALS inhibitors [10, 12, 14, 39]. The novel data in this work come from the experiments conducted with [^{14}C]-Tri, detecting more translocation in resistant than in susceptible plants, towards other organs and tissues, including the meristems where ALS inhibitors exert most of their action. Hyper-accumulation of carbohydrates in susceptible *Pisum sativum* L. leaves treated with ALS inhibitors has been reported [40], suggesting that ALS inhibitors affect the transport of assimilates into the phloem [41]. On these bases, perhaps this is the explanation for lower [^{14}C]-Tri translocation in susceptible plants. However, as differences in translocation were observed from 48 hours after treatment on, this is most likely due to differences in metabolism between susceptible plants that were severely affected by the herbicide and resistant plants that were much less affected. Finally, in agreement with previous studies [10], minimum [^{14}C]-Tri root translocation was detected in the corn poppy roots of all populations.

The [^{14}C]-Tri translocation pattern in R-703 plants resulted controversial because it was similar to those observed for ALS resistant populations (R-213 and R-114). What marked R-703 plants different from the other populations was that these plants were only resistant to 2,4-D. Only one plant out of 57 in this research, and out of 106 in further analyses (unpublished data), presented a mutation in the Pro197 position). Data suggested that tribenuron-methyl phytotoxicity in R-703 plants was not evolving as in susceptible plants (S-013), almost during the 96 hours following the herbicide application. Moreover, four plants without any mutation that survived the imazamox application had baffling results for R-703.

Metabolism studies with imazamox demonstrated the presence of a hydroxy metabolite, at least in one of the studied populations, R-313, with multiple resistance to ALS inhibitors and synthetic auxins. This is the first direct evidence of the presence of a NTSR mechanism in corn poppy, due to enhanced metabolism of an ALS inhibitor, which has been detected in very few dicot weed species from *Brassicaceae* family [9][42]. Metabolites were not detected in the other populations probably because enhanced metabolism was not high enough due to plant size or short evaluation times. For example, susceptible population was moving a significant imazamox amount to roots while almost none was detected in the other populations. Moreover, it is important to notice that the R-313 population showing enhanced metabolism at 72 HAT also had the significant highest [^{14}C]-Tri transport to shoots at that evaluation time. Therefore, the presence of NTSR mechanisms through enhanced herbicide metabolism, may explain not only [^{14}C]-Tri translocation patterns in this population, but translocation patterns in the R-703 population (only synthetic auxin resistant), and that some plants survived imazamox treatments being Pro/Pro for the ALS gene. Moreover, ALS gene sequencing demonstrated that TSR and NTSR mechanisms can be found in the same plants in R-313 population. However, it is impossible to disentangle if NTSR mechanisms for ALS inhibitors are directly related to 2,4-D resistance, the other way round or both.

5. Conclusions

In this study, three populations were multiple resistant while one population was only resistant to synthetic auxins. Substitutions at Pro197 endowed for a high resistance response to tribenuron-methyl, and moderate or no resistance to other non-SU ALS inhibitors. Non-target-site resistance mechanisms affecting sulfonylurea herbicides did not become evident under the strong resistance conferred by any amino-acid substitution at Pro197 to this chemical group. However, tribenuron-methyl translocation patterns in multiple resistant and only 2,4-D resistant populations suggested the presence of NTSR mechanisms. For non-SU ALS inhibitors, the presence of these NTSR mechanisms became more evident, as plants with the same genotype did not express the same phenotype. This was especially true for the IMI imazamox, where non-mutated plants were able to survive its application. Moreover, metabolism studies with imazamox confirmed the presence of enhanced metabolism at least in one *Papaver rhoeas* population for the first time. Therefore, selection pressure with ALS non-SU inhibitors has the risk to promote the evolution of NTSR mechanisms in corn poppy s. It is unknown if those mechanisms affect other modes of action, which are crucial for the management of herbicide resistance. The results exposed in this work will help in the development of future experiments aimed at disentangling the relationship between the

ALS inhibitors and the synthetic auxins resistant response, and to deepen in the NTSR mechanisms to both modes of action.

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Table1. Location and date of collection of corn poppy (*Papaver rhoeas*) populations used in the experiments.

Code	Location	Sampling location Latitude	Longitude	Year collected	Herbicide management in the field during preceding years
S-013	--	--	--	2008	Susceptible standard population obtained from Herbiseed (Herbiseed,Twyford, UK).
S-012	Belorado (Burgos)	42º24’57.8”N	3º10’49.3”W	2013	Susceptible standard population collected in a non-treated zone, far from fields.
R-213	Baldomar (Lleida)	41°54’39.0”N	1°00’21.2”E	2013	Florasulam plus 2,4-D in post-emergence.
R-313	Tosantos (Burgos)	42°24’43.7”N	3°14’39.9”W	2013	Aminopiralid plus florasulam, bifenox plus isoproturon and bromoxinil plus ioxinl plus MCPP in post and early post-emergence.
R-114	SantAntolí (Lleida)	41°37’58.4”N	1°19’44.6”E	2014	Iodosulfuron-methyl plus mesosulfuron-methyl and florasulam plus 2,4-D in post-emergence.
R-703	Almacelles (Lleida)	41°43’39.6”N	0°27’29.5”E	2003	Reported 2,4-D control failure in previous years.

Table 2. Herbicide used in dose-response experiments and alternative herbicide treatments.

Herbicide active ingredient.	Commercial product	Field rate(g a.i.·ha^{-1})	Manufacture	Dose rate used (g a.i.·ha^{-1})	
Tribenuron-methyl	Granstar 50 SX	18.7	DuPont	*R*	1200, 600, 150, 75, 37.5, 18.7, 9.3, 4.6 and 0
				S	18.7, 9.3, 4.6, 2.3, 1.1, 0.5, 0.25 and 0
Florasulam	Nikos	7.5	Dow AgrosciencesIberica	*R*	480, 240, 60, 15, 7.5, 3.7, 1.8, 0.9 and 0
				S	7.5, 3.7, 1.8, 0.9, 0.4, 0.2, 0.1 and 0
Imazamox	Pulsar 40	50	BASF España	*R*	3200, 1600, 400, 100, 50, 25, 12.5, 6,2 and 0
				S	50, 25, 12.5, 6.2, 3.1, 1.5, 0.7 and 0
2,4-D	Esteron 60	600	Dow AgrosciencesIberica	*R*	4800, 1200, 600, 300, 150, 75 and 0
				S	600, 300, 150, 75, 37.5, 18.7, 9.3 and 0

Table 3. Equation parameter of the log-logistic model used to estimate the GR_{50} of tribenuron-methyl, florasulam, imazamox and 2,4-D in S-013, S-012, R-213, R-313, R-114 and R-703 populations of corn poppy (*Papaver rhoeas*).

Biotype	GR_{50} ± SE (g a.i.·ha^{-1})[a]	*b*± SE[b]	Res SS[c]	RI[d]
		Tribenuron-metil		
S-013	0.1 ± 0.0	0.5 ± 0.1	5171	--
S-012	0.1 ± 0.0	0.6 ± 0.2	2689	0.6
R-213	25 ± 6.4	0.6 ± 0.1	10084	286
R-313	61 ± 12	0.6 ± 0.1	22189	695
R-114	31 ± 8.1	0.6 ± 0.1	10609	351
R-703	0.2 ± 0.0	0.5 ± 0.1	328	2
		Florasulam		
S-013	0.2 ± 0.0	0.7 ± 0.1	21738	--
S-012	0.4 ± 0.1	0.9 ± 0.2	8530	2
R-213	3.9 ± 0.4	2.0 ± 0.4	3899	24
R-313	2.9 ± 0.7	0.6 ± 0.1	2311	18
R-114	2.9 ± 0.3	0.9 ± 0.1	1529	18
R-703	0.4 ± 0.1	1.3 ± 0.4	1704	2
		Imazamox		
S-013	0.6 ± 0.1	0.8 ± 0.2	8917	--
S-012	0.2 ± 0.1	0.4 ± 0.1	2428	0.5
R-213	18 ± 1.0	4.3 ± 1.2	4534	30
R-313	24 ± 3.5	1.8 ± 0.4	6544	40
R-114	15 ± 1.0	1.2 ± 0.1	966	24
R-703	4 ± 0.6	1.5 ± 0.3	1098	6
		2,4-D		
S-013	69 ± 10	1.1 ± 0.2	23693	--
S-012	71 ± 24	0.8 ± 0.2	10303	1
R-213	817 ± 96	1.3 ± 0.2	2872	12
R-313	1238 ± 436	0.8 ± 0.3	18435	18
R-114	926 ± 156	1.0 ± 0.3	5038	13
R-703	1040 ± 402	0.7 ± 0.2	8399	15

[a]GR_{50}, herbicide concentration for 50% reduction of corn poppy dry weight.

[b]Slope at the GR_{50}

[c]Res SS, residual sum of square.

[d] RI (resistance index) = GR_{50}(Population) ÷ GR_{50}(S-013).

Table 4. Herbicide sensitivity to three ALS inhibitors applied at the field rate and ALS alleles identified (first column) at position 197 in five different corn poppy (*Papaver rhoeas*) populations (three multiple resistant, one synthetic auxin resistant and one susceptible). No mutations were found in position Trp574. Numbers represent sum of plants of a particular genotype with a particular phenotypic response to each herbicide applied.

	[a]R-213						R-313						R-114							R-703				S-013			
	[b]Tri	Flo			Ima		Tri	Flo		Ima			Tri		Flo			Ima		Tri	Flo	Ima		Tri	Flo	Ima	
	[c]R	R	r	S	R	r	R	r	S	R	r	S	R	S	R	r	S	R	r	S	S	r	S	S	S	r	S
Pro/Pro									1			1		3			3			17	17	4	12	17	17	1	16
Ala/Pro							1																				
Leu/Leu	2		3		2	1		1																			
Leu/Pro			1		1																						
Ser/Ser	3	3			2	2	2						2		1	1			1								
Ser/Pro	8	1		1			2				2		3			1			1								
Ser/Arg							1																				
Thr/Thr							3	5		1			5			1	1	3				1					
Thr/Pro							2		1	2	1		1			1	2	3	1								
Ser/Thr							4	6	1	5	3		2			3	1	1	2								
Ser/Leu	4	1	7		6	2	1									1											
Thr/His							1																				
Thr/Leu													1				1	3	2								
Thr/Arg									2	1	1																
Leu/Arg						1																					

[a] Corn poppy population.

[b] Herbicide applied, tribenuron-methyl (Tri), florasulam (Flo) and imazamox (Ima).

[c] Herbicide response to ALS inhibitors. R, resistance; r, moderately resistance (re-growth) and S, susceptible. For every product, only reported responses have been represented.

Table 5. Correlation between observed individual response to imazamox or florasulam and individual nucleotide and amino-acid sequence of corn poppy (*Papaver rhoeas)* acetolactate synthase. Only positions related with ALS resistance (Ala122 ,Pro197, Ala205, Asp376, Trp574, Ala653 and Gly654) and those positions where mutations were found (Glu427 and Leu648) have been represented. All sequences were compared with the wild type corn poppy ALS gene (GenBank: AJ577316). In sensitive ALS corn poppy, codon 653 encodes an alanine and not a serine residue as in other species.

Code	Ala122	Pro197	Ala205	Asp376	Trp574	Glu427	Leu648	Ala653	Gly654	Response
Wild type ALS	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	--
Imazamox										
S-013[a] (21)[b]	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
S-013 (22)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	S
R-703 (24)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703 (29)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703(30)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703 (21)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	S
R-114 (26)	GCA	*MYT*	GCA	GAT	TGG	*RAA*	*TYG*	GCT	GGT	R
R-114 (25)	GCA	*MYT*	GCA	GAT	TGG	*RAA*	TTG	GCT	GGT	r
Florasulam										
R-114 (20)	GCA	*ACT*	GCA	GAT	TGG	*AAA*	TTG	GCT	GGT	r
R-114 (17)	GCA	*ACT*	GCA	GAT	TGG	*AAA*	TTG	GCT	GGT	S

[a] Population code.

[b] Code of the sample within the population

* indicate mutated residue nucleotide

Table 6. Amount of imazamox and its hydroxy metabolite (μg·g^{-1} plant, n = 3 replicates) detected by liquid chromatography–DAD in extracts of plants from one susceptible (S-013) and three resistant (R-213, R-313 and R-703) corn poppy (*Papaver rhoeas*) populations, evaluated at 24, 48 and 72 hours after foliar application (HAT). ND: not detected; Standard error of the mean in parenthesis.

		Imazamox[a]		Hydroxy metabolite	
HAT	Population	Aerial part	Root	Aerial part	Root
24	S-013	63.6a (2.7)	ND	ND	ND
	R-213	59.0a (1.5)	ND	ND	ND
	R-313	56.6a (0.8)	ND	ND	ND
	R-703	-*	-*	ND	ND
48	S-013	90.8a (1.9)	8.7 (0.4)	ND	ND
	R-213	66.1b (2.9)	ND	ND	ND
	R-313	65.2b (2.6)	ND	ND	ND
	R-703	59.3b (3.5)	ND	ND	ND
72	S-013	123.2a (3.45)	23.6a (1.0)	ND	ND
	R-213	91.1b (1.2)	0.9b (0.1)	ND	ND
	R-313	72.0c (1.0)	ND	19.8 (1.0)	0.9 (0.0)
	R-703	97.9b (1.7)	ND	ND	ND

[a] Means within a column and evaluation time followed by the same letter are not significantly different at the 5% level as determined by the Tukey test.

* samples lost.