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

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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.

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

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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 110° 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 48 h, 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 −20 °C until use.

Mutations conferring ALS resistance in corn poppy at Pro197 and Trp574 codons were analyzed first for all the samples. Fragments of the ALS gene that included the regions of those codons were amplified

Table 1
Location and date of collection of corn poppy (*Papaver rhoeas*) populations used in the experiments.

Code	Sampling location			Year collected	Herbicide management in the field during preceding years
	Location	Latitude	Longitude		
S-013	–	–	–	2008	Susceptible standard population obtained from Herbiseed (Herbiseed,Twyford, UK).
S-012	Belorado (Burgos)	42°24′57.8″N	3°10′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.