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Resistance Mechanisms to 2,4-D in Six Different Dicotyledonous Weeds Around the World

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Abstract: 2,4-D resistance is increasing around the world due to both transgenic crops and resistance to other herbicides. The objective of this study was to characterize the currently unknown mechanisms of 2,4-D resistance in five weed species from around the globe: *Amaranthus hybridus* (Argentina), *Conyza canadensis* (Hungary), *Conyza sumatrensis* (France), *Hirschfeldia incana* (Argentina) and *Parthenium hysterophorus* (Dominican Republic), using *Papaver rhoeas* (Spain) as a standard resistant (R) species. Dose-response trials using malathion and absorption, translocation and metabolism experiments were performed to unravel the resistance mechanisms. R plants produced at least 3-folds less ethylene than susceptible plants, confirming the resistance to 2,4-D, together with resistance factors >4. *A. hybridus*, *P. hysterophorus* and *P. rhoeas* showed both reduced translocation and enhanced metabolism. In the two *Conyza* spp., the only resistance mechanism found was enhanced metabolism. Malathion synergized with 2,4-D in all these species, indicating the role of cytochrome P450 in the herbicide degradation. In *H. incana*, reduced translocation was the only contributing mechanism to resistance. Among the six dicotyledonous weed species investigated, there was a differential contribution to 2,4-D resistance of enhanced metabolism and reduced translocation. Thus, extrapolating 2,4-D resistance mechanisms from one weed species to another is very risky, if even related.

Keywords: *Amaranthus hybridus*; *Conyza* sp.; cytochrome P450; enhanced metabolism; *Hirschfeldia incana*; *Papaver rhoeas*; *Parthenium hysterophorus*; reduced translocation

1. Introduction

Commercially released in 1946, 2,4-D was used principally to control a wide spectrum of dicotyledonous weeds [1]. It is one of the oldest herbicides and its appearance revolutionized the discipline of weed science by introducing a new control method [2]. 2,4-D still remains one of the most commonly used herbicides in the world as a consequence of its low cost, selectivity, efficacy and wide spectrum of weed control [1,2]. According to its site of action (SoA), this molecule belongs to the group of synthetic auxin herbicides (SAH) (HRAC group O; WSSA Group 4) along with dicamba, picloram and others, which mimic the naturally occurring plant hormone indole-3-acetic acid [3]. 2,4-D is

a phenoxy-carboxylate among the five chemical families within SAH. It is available in several different formulations of the ester, amine salt and acid. The dimethylamine salt and the ester account for around 95% of all formulations sold globally [4].

SAH action is given by three ways: 1, altering the plasticity of the cell walls; 2, influencing the amount of protein production; and 3, increasing ethylene production [5]. All these effects can cause the plant death. The efficacy of 2,4-D as herbicide depends on several factors that involve uptake and translocation to the plant meristems [5,6]. The complexity surrounding SAH to understand their SoA is due to the involvement of large protein families in auxin perception, signaling, transport and metabolism [7]. Because its precise SoA is not well established to date, the mechanisms involved in this resistance still remain poorly understood [8]. The first cases of 2,4-D resistance were reported in *Daucus carota* and *Commelina diffusa* in 1957 [9,10]. Besides its extended use after >70 years, few cases of resistant (R) weeds to this SoA have been reported compared to others [11]. To date, 41 species have developed resistance to auxinic herbicides in 22 countries [11].

2,4-D can be absorbed through roots, stems and leaves, but due to its foliar mode of application, its uptake is mainly by leaves [5]. 2,4-D uptake and translocation is representative of the active phloem loading pathway [3]. In this sense, reduced SAH absorption and/or translocation has been related to the resistance response in several dicotyledonous weeds species [12]. More recently, the lack of 2,4-D translocation has also been reported in R biotypes of *Papaver rhoeas* [13], *Sisymbrium orientale* [14] and *Raphanus raphanistrum* [15].

2,4-D metabolism in plants can follow three paths: first, primarily through direct conjugation, leading to phytotoxic metabolites usually in susceptible (S) dicotyledonous; second, ring hydroxylation, mediated by cytochrome P450 (P450) [16], which leads to a non- or partially phytotoxic metabolite usually in tolerant monocots; and as a third option, but less common, it can be metabolized by side-chain cleavage [2]. In this regards, potential detoxification of SAH, as a non-target-site resistance (NTSR) mechanism, can contribute to resistance in several dicotyledonous weeds species, such as *Amaranthus tuberculatus*, *Carduus nutans*, *Kochia scoparia*, *Stellaria media* and *Papaver rhoeas* for 2,4-D, or *Sinapis arvensis* for dicamba. In the case of *A. tuberculatus* and *P. rhoeas*, malathion synergized with 2,4-D, indicating the potential role of P450 [17,18].

SAH have several target sites within plants: a nuclear auxin receptor proteins family (TIR1 and Auxin F-Box proteins, AFB1–5), an auxin cell influx carrier (AUX1) and two auxin efflux carrier proteins families (PIN and ABCB proteins) [8]. These potential multiple sites of action of SAH could explain the rarity of target-site resistance (TSR) mechanisms described so far in weeds, among other factors [1]. For the first time, the presence a mutation of the AUX1 gene in *K. scoparia* has only very recently been fully demonstrated as a TSR mechanism conferring cross-resistance to 2,4-D and dicamba [19].

When 2,4-D binds with its nuclear receptor TIR1, it promotes a cascade of processes, resulting in derepression of auxin-regulated genes that in turn leads to the physiological and morphological events associated with its action [20]. Among those, the enhanced expression of the ACC synthase, which stimulates ethylene production, usually in S but not in R plants, is well acknowledged [21]. Though ethylene biosynthesis it is not necessarily related to the nature of the resistance mechanism involved, it can be used as a fast screening to detect resistance to 2,4-D and other SAH [13,22].

Between 1980 and 2009, 0.8 resistance cases per year to SAH were reported, while from 2010 until today, the rate has increased to 1.4 cases per year [11]. It is expected that the number of cases will increase through the next years due to both new uses of 2,4-D enabled by the introduction of 2,4-D R crops, and also its incremental use due to greater dependence on SAH in response to the emergence of resistance to other herbicides [23]. Among many countries, very important new weeds R to 2,4-D have been cited: *Amaranthus hybridus* from Argentina [24] and *Parthenium hysterophorus* from Cuba [25], in which enhanced metabolism seems to be involved, and *Papaver rhoeas* is now spreading in France and Italy [26]. Other cases have been reported but not yet investigated, including *Conyza canadensis* from Hungary and *C. sumatrensis* from France, both found in vineyards, and *Hirschfeldia incana* from Argentina in winter cereals, reported by the co-author Dr. Vigna [11].

The aim of the current study was to characterize the currently unknown mechanisms of evolved 2,4-D resistance in the above-mentioned species *Amaranthus hybridus* from Argentina, *Conyza canadensis* from Hungary, *Conyza sumatrensis* from France, *Hirschfeldia incana* from Argentina and *Parthenium hysterophorus* from the Dominican Republic, using *Papaver rhoeas* from Spain as a standard R species. This knowledge should be useful to develop strategies to ameliorate 2,4-D resistance in these weed species.

2. Materials and Methods

2.1. Chemicals

A 2,4-D of formulated product (ester, Esteron 60[®] (EC, 60% w/v), Dow Agrosiences Iberica, Pozuelo de Alarcón, Spain) was used in greenhouse tests and laboratory studies. The analytical grade (>99.5%) was used to determine the herbicide effects on physiological and biochemical studies in plants. ¹⁴C-2,4-D acid (specific activity 2.035 GBq mmol⁻¹) with 95% radio-chemical purity was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA).

2.2. Plant Material

The seeds of original field evolved 2,4-D R populations were harvested from six different countries from Latin America and Europe in 2017 (Table 1). These populations displayed a survival of >90% at the recommended field dose (480 g ai ha⁻¹) for *Amaranthus hybridus*, *Hirschfeldia incana* and *Parthenium hysterophorus*, or at 600 g ai ha⁻¹ for *Conyza canadensis* and *Papaver rhoeas*, while it was ~25% for *Conyza sumatrensis* (data not shown). 2,4-D S populations belonging to these species were originally harvested from areas close to the fields evolving resistance to this herbicide, except for *P. rhoeas* in which a known S standard was used [13,27]. Seeds were maintained at 4 °C in a cold chamber and they had more than an 80% germination rate during this study.

Table 1. Most important characteristics of the dicotyledonous species resistant (R) to 2,4-D studied.

Species	Family	Country/Region	Crop	Survived Field Dose (g ai ha ⁻¹)
<i>Amaranthus hybridus</i>	Amaranthaceae	Argentina/Colonia Marina	Soybean	480
<i>Conyza canadensis</i>	Asteraceae	Hungary/Badacsony	Vineyard	600
<i>C. sumatrensis</i>	Asteraceae	France/Jonquieres	Vineyard	600
<i>Hirschfeldia incana</i>	Brassicaceae	Argentina/Buenos Aires	Wheat	480
<i>Parthenium hysterophorus</i>	Asteraceae	Dominican R./Basima	<i>Citrus sinensis</i>	480
<i>Papaver rhoeas</i>	Papaveraceae	Spain/Baldomar	Winter cereal	600

2.3. Ethylene Production Fast Screening

The ethylene biosynthesis stimulation could be considered to be a fast screening to detect resistance to 2,4-D and other SAH [13,22]. 480 g ai ha⁻¹ of 2,4-D was applied on R and S plants of each dicotyledonous species tested (Table 1). The experiment was performed using 10 replicates (one plant per pot) at the growth stage of four leaves. The herbicides were applied in a laboratory chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 250 L ha⁻¹ at the height of 50 cm from plant level. Twenty-four h after treatment (HAT), seedlings were excised and 400 g shoot fresh weight was placed into 10 mL syringe with 1 mL distilled water and sealed [28]. The syringes were placed in a dark incubator at 27 °C for 4 h and 1 mL of the headspace gas was analyzed for ethylene (C₂H₄) by gas chromatography [28]. The C₂H₄ was expressed as a µL per gram of fresh weight by h. The experiment was repeated twice.

2.4. Response of Dicotyledonous Weed Species to 2,4-D Pre-Treated with or without Malathion

Seeds of R and S populations of the six dicotyledonous weed species were germinated on moistened filter paper in Petri dishes, and one week later, seedlings were planted in 8 × 8 × 10 cm

pots (one plant per pot). After two weeks when R and S plant populations were at the 4–6 leaf stage, they were sprayed with 2,4-D at different doses (0, 45, 90, 180, 360, 720 and 1440 g ai ha⁻¹) as shown above. This dosage was determined in a preliminary test carried out at doses of 50, 300 and 600 g ai ha⁻¹ in the R and S populations of the six dicotyledonous species. Above-ground dry weight per plant was determined 28 days after treatment (DAT), and data were expressed as the percentage of the untreated control.

Malathion has previously been shown to inhibit 2,4-D metabolism in R *P. rhoeas* and *A. tuberculatus* by inhibiting P450 [17,18]. Seedlings of R and S populations for the six weed species were treated with malathion at 2000 g ai ha⁻¹ at the 4–6 leaf stage and then they were dried for 2 h at laboratory temperature (24 °C). After that, 2,4-D was applied at doses as shown above. The experiments were repeated twice with three replicates (ten technical replications for each population).

2.5. ¹⁴C-2,4-D Absorption and Translocation in Dicotyledonous Weeds

The ¹⁴C-2,4-D absorption and translocation assays were carried out as described by Rey-Caballero et al. [13] and Bracamonte et al. [29], with some modifications. ¹⁴C-herbicide was mixed with commercial formulations of 2,4-D at different field doses for each species (Table 1). The final 2,4-D concentrations were 480 g ai ha⁻¹ (*A. hybridus*, *H. incana* and *P. hysterophorus*) and 600 g ai ha⁻¹ (*C. canadensis*, *C. sumatrensis* and *P. rhoeas*) in 250 L ha⁻¹ with a specific activity of 0.834 kBq µL⁻¹. Plants of the R and S populations at the four leaf stage were treated with a drop (1 µL plant⁻¹) of radiolabeled solution on the adaxial surface of the second youngest leaf. The herbicides were applied in separate experiments. There were five repeats and each experiment was arranged in a completely randomized design.

The plants were harvested at 96 HAT. Previous experiments with different dicotyledonous weeds, such as *P. rhoeas*, established this time point to be the most suitable for finding maximum differences between S and R populations to SAH [13,27,30,31]. The unabsorbed ¹⁴C-herbicide was removed from the treated leaf with a water-acetone solution (1:1 v/v) by washing the plants three times separately with 1 mL of that solution. The rinse solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard, Groningen, The Netherlands) and measured by liquid scintillation spectrophotometry (LSS) using a Beckman LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA). The whole washed plants were removed from the pot and sectioned into treated leaves, the rest of the plant and roots. The plant sections were individually stored in cellulose cones for combustion, dried at 60 °C for 96 h and burned using a biological oxidant (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA, USA). The CO₂ released from combustion was captured in 18 mL of a mixture of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each sample was measured for 10 min by LSS. The radioactive values of absorption and translocation of ¹⁴C were expressed as a percentage of the total ¹⁴C-herbicide applied and absorbed, respectively.

2.6. 2,4-D Metabolism in Dicotyledonous Weeds

Seedlings from S and R populations at six true leaves of development (4–6 cm) were treated with 2,4-D doses, at 0 and 480 g ai ha⁻¹ and 600 g ai ha⁻¹, which are the field recommended rates in Latin America and Europe, respectively (Table 1), as described above for the dose-response experiments. Five plants from each population and dose were harvested at 96 HAT. As abovementioned in the preceding experiment, this time point was established according to previous research [18,27]. Plants were separated into aerial part (leaves and shoots) and root system, and were rinsed using distilled water to remove unabsorbed herbicide. They were rapidly frozen in liquid nitrogen and then stored at −80 °C until use. For these assays, the methodology described by Torra et al. [18] and Mora et al. [25] was followed using a Gold LC System from Beckman Coulter (Fullerton, CA, USA) equipped with a diode array detector (wavelength range 190–600 nm). The 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), furnished with a 4.6 mm SecurityGuard[™] ULTRA cartridges.

Quantification of 2,4-D and its metabolites was based on the calibration curve of 2,4-D. The results were expressed as percentage of 2,4-D and its metabolites. The experiment was performed for each dicotyledonous species (R and S populations) and was repeated twice at different times in the laboratory.

2.7. Data Analysis

The amount of herbicide causing a 50% reduction in dry weight compared to the untreated control (GR_{50}) was calculated by submitting the percentage data to a non-linear regression analysis using a log-logistic model of three parameters (four parameters ($Y = c + \{(d-c)/[1 + (x/g)^b]\}$), where c and d are the upper and lower asymptotic limits, b is the slope, g is the GR_{50} and x is the herbicide concentration) [32]. The three-parameter model assumes that the lower limit is zero. Regression analyses were performed in SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA) with the R program. The resistance factor (RF) was computed as $GR_{50} (R)/GR_{50} (S)$ [29].

Data from ethylene production, absorption and translocation, as well as metabolism experiments, were submitted to an analysis of variance (ANOVA) in a completely randomized design for each species, comparing R and S populations. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected for all tests. Values of $p < 0.05$ were considered statistically significant and mean comparisons were performed using the Tukey's test at the 5%. Statistical analyses were conducted with the Statistix 9.0 software (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Ethylene Production Fast Screening

Foliar application of 2,4-D stimulated ethylene production in all plants studied (both R and S for each species), but the comparison between R and S populations showed always significant differences in all species. S plants produced between three- and nine-folds more ethylene than R plants (Figure 1). When comparing the S populations across the species, there were no differences in the production of ethylene in S plants of *A. hybridus*, *C. canadensis*, *C. sumatrensis*, *P. hysterophorus* and *P. rhoeas*, while *H. incana* produced roughly two-folds less ethylene. On the other hand, *C. sumatrensis* R plants produced more ethylene than the rest of the species.

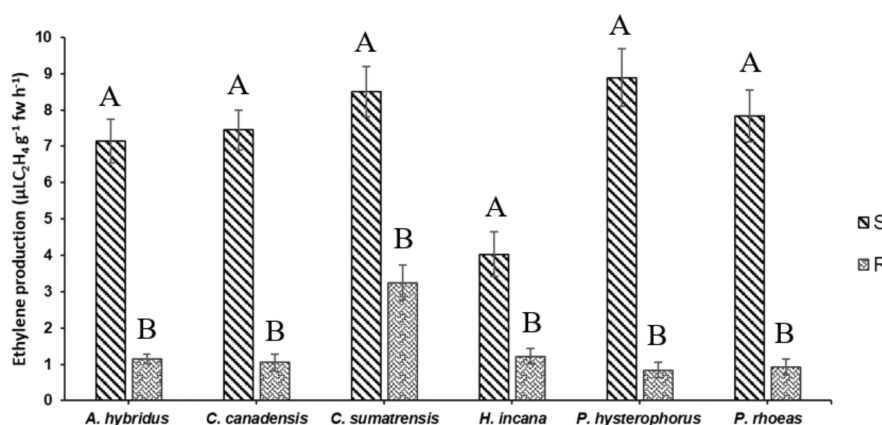


Figure 1. Ethylene production induced by 2,4-D at 480 g ai ha⁻¹ in different dicotyledonous weed species resistant (R) and susceptible (S) populations. Means followed by the same letter within each species do not differ by the Tukey test ($p < 0.05$). Vertical bars are means $\pm$ the standard errors of the mean ($n = 10$).

3.2. Response of Dicotyledonous Weed Species to 2,4-D Pre-Treated with or without Malathion

All R and S populations of the six species had a general common behavior after the first days of 2,4-D application in the preliminary test: appearance of epinasty, growth reduction and morphological damage at high doses (600 g ai ha⁻¹) in the five R populations and at low doses (50 g ai ha⁻¹) in the six

S populations. The R-*C. sumatrensis* population suffered these deformations at doses of 300 g ai ha⁻¹. While all S populations died 21 DAT at the dose of 300 g ai ha⁻¹, the R populations survived at the dose of 600 g ai ha⁻¹ except for *C. sumatrensis* that died. The GR₅₀ values clearly showed 2,4-D resistance of the species *A. hybridus*, *C. canadensis*, *H. incana*, *P. hysterothorus* and *P. rhoeas*, while the case of *C. sumatrensis* was special because although it had an RF greater than 4, the plants did not survive the field dose (Table 2).

Table 2. Parameters of the log-logistic used to estimate the dose-response regression curves (% dry weight) in dicotyledonous weeds susceptible (S) and R to 2,4-D with (+malathion) or without (-malathion) previous application of malathion at 2000 g ai ha⁻¹.

Species	Population/ ±Malathion	d	b	GR ₅₀ (g ai ha ⁻¹)	RF	p-Value
<i>A. hybridus</i>	R-malathion	99.09	2.72	572.62 ± 34.17	6.06	<0.001
	R+malathion	99.97	2.25	294.10 ± 8.77	3.26	<0.001
	S-malathion	100.25	2.35	94.41 ± 2.49	-	<0.001
	S+malathion	100.49	2.51	90.12 ± 6.11	0.95	<0.001
<i>C. canadensis</i>	R-malathion	100.61	3.12	797.25 ± 31.21	16.36	0.018
	R+malathion	100.21	2.67	55.91 ± 3.52	1.14	<0.001
	S-malathion	100.02	3.49	48.71 ± 4.82	-	<0.001
	S+malathion	100.01	3.42	44.95 ± 4.85	0.92	<0.001
<i>H. incana</i>	R-malathion	101.11	3.56	563.06 ± 15.55	4.17	<0.001
	R+malathion	100.94	3.43	553.46 ± 13.65	4.10	<0.001
	S-malathion	99.11	2.36	134.74 ± 4.66	-	<0.001
	S+malathion	98.69	2.84	140.91 ± 7.23	1.04	0.019
<i>P. hysterothorus</i>	R-malathion	100.53	3.10	847.65 ± 30.46	9.95	<0.001
	R+malathion	98.95	2.03	386.03 ± 12.97	4.53	<0.001
	S-malathion	100.39	2.47	85.15 ± 3.61	-	<0.001
	S+malathion	100.52	2.46	86.85 ± 2.65	1.01	<0.001
<i>P. rhoeas</i>	R-malathion	100.71	2.71	875.36 ± 22.06	11.03	<0.001
	R+malathion	100.50	2.08	399.14 ± 12.92	5.03	<0.001
	S-malathion	99.75	1.76	79.34 ± 2.33	-	<0.001
	S+malathion	99.88	2.73	83.26 ± 3.43	1.04	<0.001
<i>C. sumatrensis</i>	R-malathion	100.07	3.21	112.28 ± 4.15	4.37	0.017
	R+malathion	100.74	1.96	27.05 ± 2.16	1.05	<0.001
	S-malathion	99.99	1.93	25.64 ± 2.29	-	0.023
	S+malathion	100.01	2.26	22.43 ± 3.06	0.87	<0.001

d is the upper limit and b is the slope of the curve; GR₅₀ is the herbicide rate inhibiting plant growth by 50% with respect to the untreated control. RF = Resistance factor = GR₅₀ of the R population/GR₅₀ of the S population. ± Standard error of the mean (n = 10).

To test the hypothesis that enhanced 2,4-D metabolism is conferred by P450, its known inhibitor malathion was tested. Pretreatment with malathion followed by 2,4-D resulted in partial reduction of RF (around 50%) in *A. hybridus*, *P. hysterothorus* and *P. rhoeas* compared to plants only treated with the herbicide. In the cases of *C. canadensis* and *C. sumatrensis*, malathion fully synergized with 2,4-D, and all R plants became S. Finally, this pretreatment had no effect on *H. incana*.

3.3. ¹⁴C-2,4-D Absorption and Translocation in Dicotyledonous Weeds

The absorption remained similar between R and S populations of all species at 96 HAT, suggesting no differences could be attributed to this factor. However, there were several differences in translocation between R and S plants of most species except for *C. canadensis* and *C. sumatrensis*, which showed similar amounts of radiolabeled herbicide retained in treated leaves (Table 3). Comparison between R and S populations of the same species showed around 25% less herbicide moved from treated leaves to the rest of the plants in *P. rhoeas* and 40% for *P. hysterothorus*, while *A. hybridus* and *H. incana* showed intermediate values around 32%.

Table 3. ^{14}C -2,4-D absorption (% of from total applied) and translocation (% from uptake) in different dicotyledonous weeds R and S populations at 96 h after treatment (HAT).

Species	Populations	Absorption	Translocation		
			Treated Leaf	Rest of Plant	Root
<i>A. hybridus</i>	R	59.1 $\pm$ 2.48 A	97.6 $\pm$ 2.54 A	1.5 $\pm$ 1.54 B	0.9 $\pm$ 0.25 A
	S	60.1 $\pm$ 2.02 A	65.3 $\pm$ 2.18 B	24.1 $\pm$ 3.04 A	10.6 $\pm$ 0.31 A
<i>C. canadensis</i>	R	69.4 $\pm$ 5.70 A	97.2 $\pm$ 2.1 A	1.8 $\pm$ 1.1 A	1.0 $\pm$ 0.3 A
	S	73.8 $\pm$ 6.3 A	98.1 $\pm$ 4.3 A	1.2 $\pm$ 0.9 A	0.7 $\pm$ 0.4 A
<i>H. incana</i>	R	64.7 $\pm$ 6.2 A	96.6 $\pm$ 2.6 A	2.3 $\pm$ 0.8 B	1.1 $\pm$ 0.5 B
	S	70.5 $\pm$ 6.9 A	64.9 $\pm$ 5.6 B	25.7 $\pm$ 2.1 A	9.4 $\pm$ 0.7 A
<i>P. hysterothorus</i>	R	62.4 $\pm$ 3.2 A	98.2 $\pm$ 2.3 B	1.2 $\pm$ 0.2 B	0.6 $\pm$ 0.2 B
	S	61.9 $\pm$ 5.6 A	58.3 $\pm$ 5.4 A	25.6 $\pm$ 2.6 A	16.1 $\pm$ 3.4 A
<i>P. rhoeas</i>	R	66.73 $\pm$ 4.2 A	95.6 $\pm$ 4.4 A	2.6 $\pm$ 0.9 B	1.8 $\pm$ 0.7 B
	S	65.8 $\pm$ 3.2 A	70.3 $\pm$ 3.2 B	23.2 $\pm$ 1.2 A	6.5 $\pm$ 0.4 A
<i>C. sumatrensis</i>	R	78.9 $\pm$ 3.2 A	96.8 $\pm$ 3.6 A	3.1 $\pm$ 1.5 A	0.1 $\pm$ 0.1 A
	S	80.1 $\pm$ 2.4 A	97.6 $\pm$ 1.8 A	1.8 $\pm$ 0.9 A	0.6 $\pm$ 0.5 A

Same letter within a column is not different by the Tukey test at 95%. $\pm$ standard error of the mean (n = 5).

3.4. 2,4-D Plant Metabolism

The total average recovery of the applied 2,4-D was 93% ($\pm$ 2.1) in both R and S dicotyledonous populations assays. After 96 HAT of application, 2,4-D remained unmetabolized in all S populations. Conversely, R plants of *A. hybridus*, *C. canadensis*, *P. hysterothorus*, *P. rhoeas* and *C. sumatrensis* had transformed between 40 and 62% of 2,4-D applied to nontoxic metabolites in foliar tissues (Table 4). In those species, greater proportions of metabolites were found in foliar tissues; however, between 0.8 and 1.9 percent of nontoxic metabolites were also detected in roots. An exception was *C. sumatrensis* where no metabolites were detected in roots. No metabolites were detected in the foliar tissues or roots of *H. incana*.

Table 4. 2,4-D metabolism (expressed as %) in different dicotyledonous weeds R and S populations at 96 h after treatment (HAT).

Species	Populations	Foliar		Root	
		2,4-D	Metabolites Non-Toxic	2,4-D	Metabolites Non-Toxic
<i>A. hybridus</i>	R	46.5 $\pm$ 4.5 B	51.8 $\pm$ 3.3	ND ^a	1.7 $\pm$ 0.4
	S	87.4 $\pm$ 3.6 A	ND	12.58 $\pm$ 1.8	ND
	p-value	0.0001	-	-	-
<i>C. canadensis</i>	R	36.2 $\pm$ 5.1 B	62.2 $\pm$ 2.9	ND	1.6 $\pm$ 0.3
	S	97.6 $\pm$ 1.8 A	ND	2.4 $\pm$ 0.9	ND
	p-value	0.0001	-	-	-
<i>H. incana</i>	R	98.4 $\pm$ 1.2 A	ND	1.6 $\pm$ 0.5 B	ND
	S	89.9 $\pm$ 2.1 B	ND	10.1 $\pm$ 1.2 A	ND
	p-value	0.0037	-	0.0143	-
<i>P. hysterothorus</i>	R	38.8 $\pm$ 6.1 B	59.3 $\pm$ 3.5	ND	0.8 $\pm$ 0.2
	S	86.2 $\pm$ 5.6 A	ND	13.8 $\pm$ 2.9	ND
	p-value	0.0010	-	-	-
<i>P. rhoeas</i>	R	38.2 $\pm$ 2.7 B	58.6 $\pm$ 2.1	1.3 $\pm$ 0.6 B	1.9 $\pm$ 0.7
	S	91.2 $\pm$ 1.4 A	ND	8.8 $\pm$ 0.8 A	ND
	p-value	0.0001	-	0.0004	-
<i>C. sumatrensis</i>	R	59.4 $\pm$ 4.3 B	39.7 $\pm$ 2.7	0.9 $\pm$ 0.2 B	ND
	S	96.9 $\pm$ 2.4 A	ND	3.1 $\pm$ 1.0 A	ND
	p-value	0.0005	-	0.0109	-

^a ND = not detected. Same letter within a column is not different by the Tukey test at 95%. $\pm$ standard error of the mean (n = 5).

4. Discussion

Resistance to 2,4-D was confirmed in all the R populations studied for the six dicotyledonous weed species, both thanks to ethylene production and dose-response experiments (Figure 1 and Table 2). The RF (always ≥ 4) were in the range reported in previous research for *A. hybridus* [24], *P. rhoeas* [13,18,27], *P. hysterothorus* [25] or close relative species to *H. incana* such as *R. raphanistrum* [21]. Also, in this work, ethylene biosynthesis supported susceptibility and resistance of all populations; all R plants tested accumulated less ethylene than S plants for each species, as in previous studies [13,28]. In this respect, S plants of *H. incana* accumulated less ethylene than the other species, which was correlated with the highest GR₅₀ (135 g ai ha⁻¹), suggesting that this species would be less S and more 2,4-D tolerant, than the rest. Overall, these results suggested a clear 2,4-D resistance in the studied species, except in the case of *C. sumatrensis* in which a slow evolution of the resistance to 2,4-D was observed, because R plants did not survive the field dose and showed the lowest reduction in ethylene production (3-folds) compared to the rest of the species.

Absorption was not related to 2,4-D resistance in the six weed species of this work, and globally, the results were comparable with other studies on these and other plant species where this NTSR mechanism played a negligible role in explaining 2,4-D resistance [8]. In very few cases, differential ¹⁴C-2,4-D absorption between S and R plants contributed to resistance, like *Glechoma hederacea* [33] and *Lactuca serriola* [30].

Altered 2,4-D translocation and increased metabolism were clearly related to resistance in this work, although, the weight of each mechanism was different among the species studied. Three species showed both mechanisms: *A. hybridus* (54% of 2,4-D was degraded), *P. hysterothorus* (61% degraded) and *P. rhoeas* (62% degraded). In all these three species, non-toxic metabolites were also found in the roots at 96 HAT. In the two *Conyza* sps., only enhanced metabolism was found: while *C. canadensis* degraded 64% of the herbicide and metabolites were detected in the roots, *C. sumatrensis* degraded only 41% of 2,4-D and metabolites were not found in the roots, again suggesting a lower degree of 2,4-D resistance evolution in this species. In all of the above-mentioned five species, malathion synergized with 2,4-D, indicating that P450 is involved in the herbicide degradation. Interestingly, in *Conyza* sps., without reduced translocation, the sensitization of R plants was full. Accordingly, 2,4-D enhanced metabolism was reported in an *A. tuberculatus* population with similar behavior, with a full phenotype reversion of the R plants with malathion, and with no differences in translocation between biotypes [17]. In *A. hybridus*, *P. hysterothorus* and *P. rhoeas*, malathion partially reversed the phenotype from R to S. Similarly, in a multiple 2,4-D and glyphosate R *P. hysterothorus* population, the same reduced translocation and partial reversion of the phenotype with malathion occurred [25]. No metabolism was detected in *H. incana*.

Two scenarios are plausible when impaired translocation and enhanced metabolism both occur, particularly when full synergism is not accomplished with a P450 inhibitor. If reduced transport was a secondary effect of metabolism and only P450 was involved in the degradation, it would be expected that malathion should fully reverse the phenotype. If not, it could be hypothesized that other degrading enzymes could be involved, as different metabolism routes for 2,4-D can be found in plants [2]. On the other hand, a second mechanism responsible of the reduced transport could be present, such as SAH influx or efflux transporters [8]. The necessity of unraveling the relationship between these two physiological processes has already been stated for *P. rhoeas* [18]. In this sense, the presence of two 2,4-D resistance mechanisms, though not metabolism, has already been appointed for other species [15]. Also, inheritance studies of SAH resistance have shown that two or several minor genes can be involved in different weeds species [34–36]. Applying the parsimony principle, reduced translocation might be a secondary effect of enhanced metabolism, but it should be further investigated together with the possible role of other enzyme families.

Metabolic resistance places a serious challenge for potential cross-resistance to other herbicides, especially to other SAH families. For example, among the species studied, cross-resistance to dicamba (benzoate) in 2,4-D R populations has already been cited for *P. rhoeas* [13], or for *A. hybridus* and

P. hystrophorus by means of enhanced metabolism [24,25]. For other species not studied here, like *A. tuberculatus* [37] or *R. raphanistrum* [21], cross-resistance to dicamba has also been demonstrated.

In this research, *H. incana* was the only species in which the only contributing mechanism to 2,4-D resistance was the reduced translocation. The absence of metabolism detected in R plants was supported by lack of effect in the inhibition of P450 by malathion. Similarly, in R biotypes of *L. serriola* and *R. raphanistrum* 2,4-D metabolism was not different compared with S populations, and showed reduced uptake [30,31]. Also, lack of 2,4-D translocation was the likely mechanism of resistance in *Sisymbrium orientale* from Australia, though metabolism was not investigated [14]. Some studies point out that influx or efflux carriers could be responsible or contribute to this impaired transport [15,19,21,31]. It is known that these transporters can be SAH targets, and thus, can confer both TSR and NTSR in weeds [8], which will depend on the species and SAH. If these carriers might be involved, it should unraveled if they are the target of the SAH, in order to understand if the mechanism is TSR or NTSR based, as might be the case for the *H. incana* population in this study.

This research represents the first report worldwide of 2,4-D resistance in the important weed species *C. canadensis*. Also, resistance mechanisms were investigated for the first time in *H. incana* and *A. hybridus*. Moreover, for *C. sumatrensis*, this is first case reporting 2,4-D resistance in Europe, while there is a single case in the rest of the world citing a multiple R biotype to five SoAs, including 2,4-D, from Brazil in 2017 [11]; however, this research represents the first one to unravel the resistance mechanisms in the species. In addition, this is the second study worldwide reporting resistance to 2,4-D in *P. hystrophorus* [25], and the first case for Dominican Republic. For *P. rhoeas*, results confirmed previous research with other populations from the same country [13,18].

5. Conclusions

Overall, across the six dicotyledonous weed species investigated, there was a differential contribution to 2,4-D resistance of the two resistance mechanisms analyzed, enhanced metabolism involving P450 and reduced translocation, while in some species both contributed, and in others, only one of them seemed to be responsible. This is in agreement with previous research in which a variety of resistance mechanisms have been described depending on the species, maybe as a result of the multi target nature of SAH [8]. The latest studies have stated the likelihood of different resistance-conferring mechanisms even within populations of the same species [15,21]. Therefore, this study also emphasizes the dangers of extrapolating 2,4-D resistance mechanisms from a few weed species to others, even if they are close relatives. Future research is required to identify the P450 genes and other enzymes potentially responsible to understand the routes of 2,4-D degradation on a species basis. Also, it is important to unravel the relationship between enhanced metabolism and reduced transport when both occur. Could impaired translocation be a secondary effect of metabolism? Or, is there another resistance mechanism also contributing to resistance? If so, or if reduced transport is the only mechanism present, like in *H. incana* in the current study, the precise mechanism should be found and its TSR or NTSR nature deciphered.

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