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Filago pyramidata Tolerant to ALS-Inhibiting Herbicides: A New Invasive Weed in Olive Groves of Southern Spain

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Abstract: Weeds that usually grow in non-agricultural areas have become increasingly common invading perennial crops. Species of the genus *Filago*, in addition to invading Spanish olive groves, have developed certain levels of natural tolerance to the acetolactate synthase (ALS) inhibiting herbicide flazasulfuron. The objective of this study was to determine the level and the mechanism involved in the tolerance to flazasulfuron in *Filago pyramidata* L., which occurs in olive groves of southern Spain, as well as to identify possible cross- or multiple-tolerances by evaluating alternative herbicides for its control. A population resistant (R) to flazasulfuron and a susceptible (S) one of *Conyza canadensis* were used as references. The accessions of *F. pyramidata* presented LD_{50} values (from 72 to 81 g active ingredient (ai) ha^{-1}) higher than the field dose of flazasulfuron (50 g ai ha^{-1}), being 11–12.5 times more tolerant than the S population of *C. canadensis*, but less than half the R population (170 g ai ha^{-1}). Enzymatically, *F. pyramidata* was as sensitive to flazasulfuron (I_{50} = 17.3 µM) as the S population of *C. canadensis*. *Filago pyramidata* plants treated with flazasulfuron, combined with 4-chloro-7-nitro-2,1,3-benzoxadiazole, had a growth reduction of up to 85%, revealing the participation of glutathione-S-transferases in herbicide metabolism. *Filago pyramidata* presented cross-tolerance to the different chemical groups of ALS inhibitors, except triazolinones (florasulam). Synthetic auxins (2,4-D and fluroxypyr) presented good control, but some individuals survived (low multiple resistance). Cellulose synthesis, 5-enolpyruvylshikimate-3-phosphate, 4-hydroxyphenylpyruvate dioxygenase, protoporphyrinogen oxidase, photosystem I, and photosystem II inhibitor herbicides, applied in PRE or POST-emergence, presented excellent levels of control of *F. pyramidata.* These results confirmed the natural tolerance of *F. pyramidata* to flazasulfuron and cross-tolerance to most ALS-inhibiting herbicides. The mechanism involved was enhanced metabolism mediated by glutathione-S-transferases, which also conferred low multiple tolerance to synthetic auxins. Even so, herbicides with other mechanisms of action still offer excellent levels of control of *F. pyramidata.*

Keywords: 4-chloro-7-nitro-2,1,3-benzoxadiazole; cottonrose; glutathione-S-transferases; herbicide metabolism; herbicide tolerance; terafit

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1. Introduction

Olive growing is a fundamental pillar in the Spanish agri-food system, having great economic, social, environmental, and territorial repercussions [1]. Olive groves occupy more than 2.75 million hectares, distributed mainly in the center-south and east of the country, which produce 70% of the olive oil in the European Union and 45% of the world [2].

Spain is the world leader in surface area, production, and foreign trade in olive oil; however, one of the biggest challenges is weed management [3], which even includes non-chemical alternatives to a greater or lesser extent [4], but the main tool for managing this vegetation continues to be herbicides. As a result of the use of these substances, various biotypes of weeds have been selected for resistance to herbicides, such as glyphosate [5]. In addition, some weed species show natural tolerance to the herbicides used and become more and more frequent dominants [6,7].

Herbicide resistance is a phenomenon that occurs with some individuals within a weed species, of which their progeny become dominant in the area after multiple applications of herbicides of the same mechanism of action. Instead, tolerance to herbicides is a characteristic of the species, regardless of whether or not they have been treated with herbicides [7].Until now, the weeds found to be resistant to herbicides in Spanish olive groves belong to genera that occur in annual and perennial crops, such as *Bromus, Conyza, Lolium, Papaver*, etc. [5]. However, intensive weed management practices have altered plant biodiversity [8], and, in recent years, species that generally occur in uncultivated areas have become more common in olive groves [9], such as species of the genus *Filago*.

Belonging to the Asteraceae family and the Gnaphalieae tribe, the *Filago* genus groups 40–45 species, of which 20 occur in the Iberian Peninsula [10]. *Filago* species are morphologically very similar to annual plants, which can make their identification difficult [10]. However, *Filago pyramidata* L. is difficult to confuse with species of the same genus, since, in the seedling stage, it is more similar to species of the genus *Allyssum* (Brasicaceae) [11]. This weed germinates in autumn-winter, forming dense rosettes, and, between March and July, it emits prostrate or erect flower stems up to 40 cm [12]. *Filago pyramidata* is widely distributed in Andalusia and grows both in open areas such as roadsides, clearings in the forest or scrub, as well as in cultivated areas [13], such as olive groves. In recent agricultural years, *F. pyramidata* have become increasingly common and abundant in olive groves in southern Spain, but, in addition, producers have observed that this weed survives field doses of flazasulfuron (50 g ai ha^{-1}), an acetolactate synthase (ALS) inhibiting herbicide.

ALS (EC 2.2.1.6) catalyzes the biosynthesis of branched-chain amino acids (isoleucine, leucine, and valine) in plants [14] by converting two pyruvate molecules to (S)-2-acetolactate or converting one pyruvate molecule and one 2-ketobutyrate molecule in (S)-2-aceto-2-hydroxybutyrate [15]. ALS inhibitors are one of the most extensive herbicidal mechanisms of action (MoA), grouping six chemical families (imidazolinones–IMI, pyrimidinyl benzoates–PYB, sulfoanilides–SA, sulfonylureas–SU, triazolinones–TZ, and triazolopyrimidines–TP) [16]. These herbicides block the channel through which the substrates access the active site of the ALS, preventing the biosynthesis of branched-chain amino acids, which result in plant death [15]. Flazasulfuron [1-(4,6-dimethoxypyrimidin-2-yl)-3-(3-trifluoromethyl-2-pyridylsulfonyl)urea] is a SU, and, after 2,4-D, it is the second most preferred herbicide for Spanish olive growers to combat glyphosate-resistant weeds [17,18] because it can be applied pre- and post-emergence (early or late). However, various weeds have quickly presented resistance or tolerance to this herbicide [18,19].

Tolerance or resistance to herbicides can be conferred by physiological, biochemical, or molecular mechanisms, which are grouped into target-site (TS–gene amplification and mutations) and non-target-site (NTS–limited uptake, impaired translocation, vacuolar sequestration, and metabolism) [20,21]. Depending on the mechanism involved, tolerance/resistance can be crossed (to herbicides with the same MoA) or multiple (to herbicides with different MoA) [22]. In this study, the objective was to characterize the susceptibility level to flazasulfuron in *F. pyramidata*, reported by olive growers from southern Spain, to unravel the possible mechanism responsible for tolerance, as well as to identify possible cross- or multiple-tolerances by evaluating alternative herbicides for its control.

2. Materials and Methods

2.1. Field Screening and Biological Material

Pre- and post-emergence field screenings with flazasulfuron (Terafit® WG 25%, *w*/*w*; Syngenta) on *F. pyramidata* plants were carried out in an olive grove located in Montizón, Jaén, southern Spain (38°20′42.3″ N, 3°08′34.6″ W). The prescreening was carried out in mid-October 2020, and the post-screening, on *F. pyramidata* plants with 6–10 true leaves, was carried out at the end of February 2021 in 4 m × 5 m plots, which included a row of olives. The flazasulfuron pre- and post-treatments were distributed in a randomized complete block design with four replicates. In addition, an untreated (UT) plot was included as a control. For both pre- and post-screening, 50 g ai ha^{-1} of flazasulfuron (field recommended dose) were applied with a backpack sprayer equipped with four 11,002 nozzles 50 cm apart, at a pressure (with CO_2) of 200 KPa to deliver 250 L ha^{-1} [23].

The efficacy was evaluated at 120 days after treatment (DAT) with a visual scale from 0 to 100% control, where the 0 corresponds to no control, and 100 corresponds to total control [24]. At the end of screening tests, 25 surviving flowering plants were collected and dried at room temperature. Ripe seeds were removed from the inflorescences, identified as PRE, POST, and UT accessions, and they were stored at 4 °C to test their tolerance to flazasulfuron in subsequent experiments. Due to the high survival rate of *F. pyramidata* plants in field screenings, and to have a reference level of susceptibility/tolerance to flazasulfuron, the H5 and H6 populations of *Conyza canadensis* were included in this study. The H5 population was characterized as being 28 times more resistant (R) to flazasulfuron than the H6 population (susceptible—S) in previous work by this research group [19].

Seeds of *F. pyramidata* and *C. canadensis* were germinated in Petri dishes on filter paper moistened with distilled water. Petri dishes were sealed with Parafilm, and then they were placed in a growth chamber at 28/18 °C (16 h day/8 h night) and intensity 350 µmol m^{-2} s^{-1}. Seedlings were individualized in 250 mL pots with sand/peat (1:2, *v*/*v*) and placed in a greenhouse at 30/18 °C (day/night) and 80% relative humidity. The plants of both species were used for the different experiments in the rosette stage with 6–10 true leaves.

2.2. Flazasulfuron Dose-Response Curves

The flazasulfuron doses tested in these experiments were: 0, 2.5, 5, 10, 20, 40, 80, 160, 240, and 320 g ai ha^{-1} for the accessions PRE, POST, and UN of *F. pyramidata* and the S *C. canadensis* population; and, they were 0, 20, 40, 80, 160, 320, 640, and 1280 g ai ha^{-1} for the R population of *C. canadensis*. Ten plants from each accession/population, chosen at random, were treated per herbicide dose in a spray chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA), equipped with a 8002E nozzle and calibrated to deliver 250 L ha^{-1} at 250 kPa at a height of 50 cm. After herbicide treatments, the *F. pyramidata* and *C. canadensis*, plants were taken and kept in a greenhouse at 30/18 °C day/night, being irrigated as necessary to maintain the field capacity of the substrate. Twenty-eight DAT is the number of dead plants were recorded, and the aerial part of each plant was cut at ground level and stored individually in paper bags, dried at 60 °C for four days, and weighed. The data of dry weight and mortality of plants were transformed to percentages, concerning the untreated control, to estimate the necessary herbicide dose to reduce the dry weight of the shoots and to kill a weed population by 50% (GR_{50} and LD_{50}, respectively).

2.3. ALS Activity Assay

Three-gram samples of young leaf tissue of *F. pyramidata* (pool of UT, PRE, and POST accessions) and R and S *C. canadensis* populations were collected, frozen in liquid N_2, and stored at −80 °C until use. The ALS was extracted according to Ref. [19]. The supernatant, containing the crude ALS extract, was immediately used for the enzyme assays and to determine the total protein content with the Bradford method [25].

The enzymatic activity of the ALS was assayed using technical-grade flazasulfuron (≥98.0% pure, Merk-Sigma Aldrich, Spain) (0, 1, 5, 10, 25, 50, 100, 200, and 400 µM for *F. pyramidata* and *C. canadensis* S; and 0, 25, 50, 100, 200, 400, 600, 800, and 1000 µM for R *C. canadensis*), obtained from a stock solution of mg mL^{-1}. The maximum specific activity of ALS (nmol acetoin mg^{-1} protein h^{-1}) was measured in the absence of the herbicide. The experiment was performed twice with five repetitions per herbicide concentration and population. Finally, the I_{50} (herbicide rate that inhibits ALS by 50%) was calculated.

2.4. Flazasulfuron Metabolism Inhibitors

Eight sets of 10 *F. pyramidata* plants were prepared for these experiments. One set of plants did not receive any treatment and was used as a control. Two sets of plants were treated with the plant metabolism inhibitors malathion (1000 g ai ha^{-1}), pyperonylbutoxide (PBO, 1000 g ai ha^{-1}), or 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl, 240 g ai ha^{-1}), i.e., six sets of plants were treated in this step. Three sets (one of each inhibitor) were used to evaluate the effect of these substances on plant growth. The other three sets of *F. pyramidata* plants received a flazasulfuron treatment (50 g ai ha^{-1}) at 3 (malathion and PBO) or 48 (NBD-Cl) h after the treatment (HAT) with the metabolism inhibitors, together with the last set of plants. The applications of malathion, PBO, NBD-Cl, and flazasulfuron were performed in the spray chamber.

After the herbicide treatment, plants were taken to a greenhouse and maintained under the conditions previously described in Section 2.2. At 28 DAT, the fresh weight of the plants was determined and converted to percentage concerning untreated control. Results were compared with the flazasulfuron test without the application of metabolism inhibitors. Experiments were performed twice at different times.

2.5. Herbicide Treatments Tested in Greenhouse

Alternative herbicides were applied to sets of 15 plants to detect cross- or multiple-resistances and to develop a potential integrated weed management (IWM) program. The different herbicides and doses used (detailed in Table 1) were applied under the same conditions and spray volume described in Section 2.2.

Table 1. Mechanism of action (MoA), active ingredient, rate (g ai ha^{-1}), application time (PRE- and POST-emergence), and trade name of the herbicides tested at a greenhouse on the population of *Filago pyramidata* L. found in an olive field from southern Spain.

MoA [1]	Active Ingredient	Rate	Time	Trade Name
ALS inhibitor	Bispiribac-sodium	30	POST	Nominee
	Florasulam	7.5	POST	Prosulam
	Flucarbazone	21	POST	Everest
	Imazamox [2]	40	POST	Pulsar 40
	Tribenuron-methyl	20	POST	Granstar
Auxin mimics	2,4-D	900 [4]	POST	U46D Complet
	Fluroxypyr	300	POST	Praxis
Cellulose synthesis inhibitor	Indaziflam	50	PRE	Alion
EPSPS inhibitor	Glyphosate	960 [4]	POST	Roundup Energy
HPPD inhibitor	Tembotrione	120	POST	Laudis
PPO inhibitor	Oxyfluorfen	240	PRE	Goal
	Oxyfluorfen	240	POST	Goal
	Flumioxazin [3]	350	PRE	51WDG Select
	Flumioxazin [3]	350	POST	51WDG Select
PSI inhibitor	Paraquat	400	POST	Gramaxone
PSII inhibitor	Atrazine	2000	POST	Gesaprim

Table 1. *Cont.*

MoA [1]	Active Ingredient	Rate	Time	Trade Name
Auxin mimics + EPSPS inhibitor	2,4-D + Glyphosate	640 [4] + 960 [4]	POST	Kyleo
ALS inhibitor + EPSPS inhibitor	Flazasulfuron + Glyphosate	50 + 960 [4]	POST	Terafit + Roundup Energy

[1] MoA: ALS–acetolactate synthase, EPSPS–5-enolpyruvylshikimate-3-phosphate, HPPD–4-hydroxyphenylpyruvate dioxygenase, PPO–protoporphyrinogen oxidase, PSI–photosystem I, PSII, photosystem II. [2] DASH 1.25 L ha^{-1}, [3] CHIDOR 2 L ha^{-1}, [4] g acid equivalent ha^{-1}.

Pre-emergence treatments were carried out on 250-mL pots containing 0.1 g of *F. pyramidata* seeds, and postemergence treatments were conducted on plants with 6–10 true leaves. The treated plants were kept in the greenhouse at 30/18 °C day/night, being irrigated as necessary. The treatments were evaluated at 28 DAT, quantifying the percentage of plant survival and fresh weight of each treatment. The experiments were repeated twice in a completely randomized design.

2.6. Analysis of Data

The values of GR_{50}, $LD_{50,}$ and I_{50} were estimated with the log-logistic equation of three parameters: $y = ([(d)/1 + (x/g)^b])$ [26], where y is the dry weight, plant mortality, or the enzymatic activity expressed in percentage in comparison with its respective untreated control; d is the upper limit; b is the slope; g is GR_{50}, LD_{50}, and I_{50} values; and x is the dose/concentration of herbicide. SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA) was used to perform the regression analyses. Resistance indexes (RI) were estimated by dividing the g values of the resistant or tolerant populations with those of the S one.

Results of flazasulfuron metabolism and alternative herbicides were submitted to ANOVA in Statistix 9.0 (Analytical Software, Tallahassee, FL, USA). Differences of $p < 0.05$ between means were considered significant and separated using the Tukey HSD test.

3. Results

3.1. Field Screening and Dose-Response

Filago pyramidata plants treated with flazasulfuron in the field at PRE and POST presented reduced growth and typical symptoms caused by sulfonylureas (SU), such as chlorosis, distortion of the leaves, and purple coloration of the veins. However, as reported by farmers, a large number of individuals in the PRE and POST plots, similar to those in the UT plots, managed to survive, complete their cycle, and produce viable seeds, denoting a certain level of innate tolerance to flazasulfuron.

In the greenhouse dose-response assays, the three accessions of *F. pyramidata* (UT, PRE, and POST) presented similar herbicide rates to cause dry weight reduction and plant mortality by 50% (Figure 1). GR_{50} values ranged from 36.1 to 44.9 g ai ha^{-1} flazasulfuron, which were closer to the GR_{50} value of the R *C. canadensis* population (55.8 g ai ha^{-1}) than to that of the S population (3.8 g ai ha^{-1}). Regarding LD_{50}, the values of the *F. pyramidata* accessions were between 11.1 and 12.5 times higher than that of the S *C. canadensis* population. In contrast, the LD_{50} of the R *C. canadensis* population was slightly more than twice (170 g ai ha^{-1}) as much herbicide was required as for *F. pyramidata* accessions (Table 2).

3.2. ALS Enzyme Activity

Because the PRE, POST, and UT accessions of *F. pyramidata* showed a similar plant mortality rate in the dose-response assays, pools of plants from the three accessions were used for this and subsequent experiments. The ALS-specific activity of *F. pyramidata* was 23–31% higher than that of the R and S *C. canadensis* populations (218–231 nmol acetoin mg^{-1} protein h^{-1}). In contrast, the ALS of *F. pyramidata* was very sensitive to flazasulfuron (similar to the S *C. canadensis* population), being inhibited by 50% only with 17.3 μM of flazasulfuron. As in previous results, the R *C. canadensis* population showed high resistance to flazasulfuron (37.9 times with respect to S population) (Figure 2, Table 3).

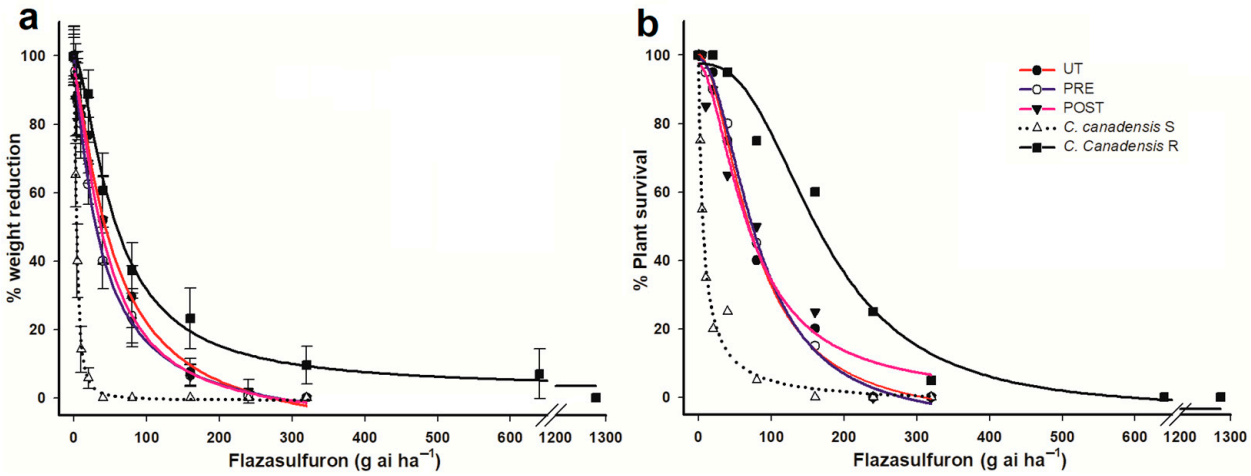

Figure 1. Flazasulfuron dose–response on dry weight reduction (**a**) and plant survival (**b**) in *Filago pyramidata* L. accessions (UT–untreated, PRE–preemergence, and POST–postemergence), collected in an olive field from southern Spain, in comparison to flazasulfuron-resistant (R) and susceptible (S) *C. canadensis* populations. Symbols denote the mean (n = 20) ± standard error.

Table 2. Mean doses (g ai ha^{-1}) of flazasulfuron required to reduce the dry weight (GR_{50}) and/or a plant population (LD_{50}) by 50% in *Filago pyramidata* L. accessions (UT–untreated, PRE–preemergence, and POST–postemergence), collected in an olive field from southern Spain, in comparison to flazasulfuron-resistant (R) and susceptible (S) *C. canadensis* populations.

Species	Accession/Population	GR_{50}	RI	LD_{50}	RI
F. pyramidata	UT	44.9 ± 5.6	11.8	75.2 ± 7.6	11.6
	PRE	36.1 ± 3.3	9.5	81.2 ± 5.1	12.5
	POST	43.8 ± 3.5	11.5	72.2 ± 5.3	11.1
C. canadensis	S	3.8 ± 0.2	—-	6.5 ± 1.4	—-
	R	55.8 ± 4.6	14.7	170.6 ± 17.4	26.2

RI = R-to-S ratio of the GR_{50} or LD_{50}. ± Confidential interval at 95% probability (n = 10).

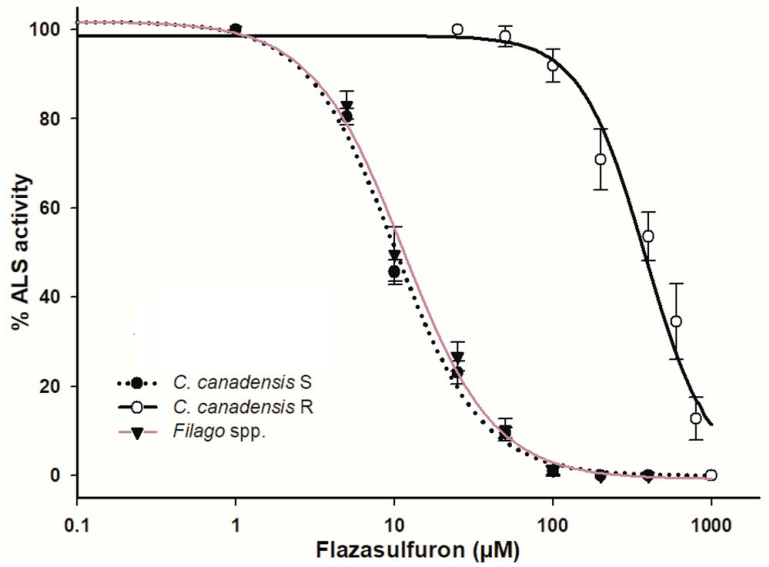

Figure 2. Log–logistic curves of the acetolactate synthase (ALS) activity in response to flazasulfuron in *Filago pyramidata* L. compared to flazasulfuron-resistant (R) and susceptible (S) *C. canadensis* populations. Symbols denote the mean (n = 3) ± standard error.

Table 3. Parameters of the log-logistic model used to estimate the concentration of flazasulfuron necessary to reduce the activity of the ALS enzyme by 50% (I_{50}) in *Filago pyramidata* L. compared to flazasulfuron-resistant (R) and susceptible (S) *C. canadensis* populations.

Population	ALS [a] Activity	d	b	R^2	I_{50}	RI
C. canadensis S	231.7 ± 11.3	99.7	1.6	0.9938	10.1 ± 0.4	—
C. canadensis R	218.4 ± 8.6	98.5	2.1	0.9598	382.9 ± 27.2	37.9
F. pyramidata	286.2 ± 7.8	100.8	1.5	0.9910	17.3 ± 1.4	1.7

[a] Nmol acetoin mg^{-1} protein h^{-1}. RI = resistant index = I_{50} (R or T)/I_{50} (S). ± Confidential interval at 95% probability (n = 3).

3.3. Inhibition of Flazasulfuron Metabolism

Filago pyramidata plants treated only with cytochrome P450 (Cyt-P450) and glutathione-S-transferases (GST) inhibitors presented a similar growth rate and fresh mass production than the untreated control (3.42 g $plant^{-1}$). In contrast, plants treated with flazasulfuron, alone or in combination with these inhibitors, produced between 37 and 85% less fresh mass than the control. Flazasulfuron alone or in combination with malathion or PBO caused a similar growth reduction in *F. pyramidata* plants; however, the treatment that caused the greatest reduction in growth and fresh mass production was the combination of the herbicide with NBD-Cl (Figure 3).

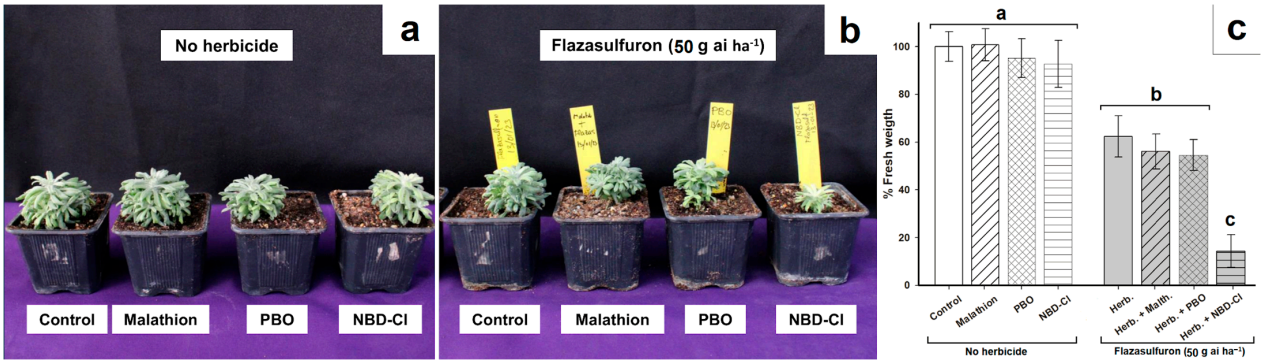

Figure 3. Qualitative (**a**,**b**) and quantitative (**c**) results of the response of *Filago pyramidata* L. plants to cytochrome P450 (malathion or PBO–piperonyl butoxide) and glutathione-S-transferase (NBD-Cl –4-chloro-7-nitro-2,1,3-benzoxadiazole) inhibitors in the absence or presence of flazasulfuron. The same letters are not different by the Tukey test at 95%. Vertical bars are the standard error (n = 20).

3.4. Alternative Herbicides Tested in Greenhouse

When evaluating recommended label rates of alternative herbicides to obtain an overview of *F. pyramidata* response in the field, a moderate to high cross-tolerance to the different chemical groups of ALS inhibitors was observed, except to TP (florasulam). The tolerance level to tribenuron-methyl (SU) was similar to that observed with flazasulfuron. Plants treated with bispiribac-sodium, flucarbazone, and imazamox presented growth reduction greater than 80%, but survival rates were greater than 60%, i.e., *F. pyramidata* presented moderate to high cross-tolerance patterns to PYB, TZ, and IMI. Synthetic auxins (2,4-D and fluroxypyr) presented good control, but few individuals were able to survive the treatment with these herbicides (low multiple tolerance). The rest of the herbicides with different MoAs (inhibitors to cellulose synthesis, EPSPS, HPPD, PPO, PSI, and PSII), applied in PRE or POST, presented excellent levels of control of *F. pyramidata* (Table 4).

Table 4. Herbicide treatment, application time (POST- or PRE-emergence), fresh weight (FW), percentage of plant survival (% Surv.), and visual effects on *Filago pyramidata* L. plants of a biotype collected in an olive field from southern Spain.

Treatment (g ai ha^{-1})	Time	FW (g plant^{-1})	% Surv.	Visual Effects
Control	—-	2.6 ± 0.4	100	—-
Bispiribac-sodium (30)	POST	0.2 ± 0.2	60	High growth reduction, but moderate cross-tolerance to PYB.
Florasulam (7.5)	POST	nd	0	Excellent control. Non-cross-tolerance to TP.
Flucarbazone (21)	POST	0.6 ± 0.3	90	High growth reduction, but high cross-tolerance to TZ.
Imazamox (40)	POST	0.2 ± 0.2	60	High growth reduction, but moderate cross-tolerance to IMI.
Tribenurom-metyl (20)	POST	1.8 ± 0.6	100	Growth reduction by 60%, but all plants survived to the SU.
2,4-D (960 [1])	POST	nd	10	Good control, few plants survived.
Fluroxypyr (300)	POST	nd	10	Good control, few plants survived.
Indaziflan (50)	PRE	nd	0	No seed germinated.
Glyphosate (960 [1])	POST	nd	0	Excellent control over 8 leaf plants.
Tembotrione (120)	POST	nd	0	Excellent control over 6 leaf plants.
Oxyfluorfen (240)	PRE	nd	0	No seed germinated.
Oxyfluorfen (240)	POST	nd	0	Slow growth reduction, but all plants died at 21 DAA.
Flumioxazin (350)	PRE	nd	0	No seed germinated.
Flumioxazin (350)	POST	nd	0	Good control, better effect in preemergence.
Paraquat (400)	POST	nd	0	Excellent control over 8 leaf plants.
Atrazine (2000)	POST	nd	0	Excellent control over 6 leaf plants.
2,4-D + Glyph. (640 [1] + 960 [1])	POST	nd	0	Synergistic effect, better and faster control than 2,4-D alone.
Flaza. + Glyph. (80 + 960 [1])	POST	nd	0	Excellent control over 8 leaf plants, synergistic effect.

[1] g acid equivalent ha^{-1}; Glyph.–glyphosate; Flaza.–flazasulfuron; nd–non-determined; PYB–pyrimidinyl benzoates; TP–triazolopyrimidines; TZ–triazolinones; IMI–imidazolinones; SU–sulfonylureas.

4. Discussion

The term 'herbicide tolerance' is used to refer to individuals of a species that are capable of surviving field doses of herbicides to which other species are susceptible [7]. Tolerant plants may or may not have been preselected with the herbicide when they survived. The PRE and POST accessions of *F. pyramidata*, screened in the field with flazasulfuron, presented survival levels similar to those of the UN accession, denoting natural tolerance to this herbicide. The dose–response assays confirmed this high level of tolerance to flazasulfuron in comparison to the S population of *C. canadensis* used as a reference, although they were low compared to the R population. *F. pyramidata* plants presented LD_{50} values higher than the field dose of flazasulfuron (50 g ai ha^{-1}). Herbicide-based weed management used in intensive production systems has caused changes in flora [8]. Weed species common in rangelands, roadsides, and other non-agricultural situations are beginning to invade annual and perennial crops, coexisting with ruderal species. Species of the genus *Centaruea* were characterized as being tolerant to ALS-inhibiting herbicides in wheat fields from the central-southern region of Spain [9], and *Carduus acanthoides* was tolerant to 2,4-D in transgenic corn and soybean production fields in Cordoba, Argentina [27]. This is a worrying phenomenon because *F. pyramidata* is a species that is invading more and more olive groves in southern Spain (De Prado, personal observation), and it could become a weed that, in combination with herbicide-resistant weeds, can make it difficult to control.

When unraveling the putative mechanisms responsible for flazasulfuron tolerance, evidence of the participation of TS mechanisms was not found, since both the basal activity and the inhibition rate of ALS in *F. pyramidata* were similar to those of the S *C. canadensis* population. Based on these results, the possibility of sequencing the *ALS* gene was ruled

out, since it is well established that mutations reduce the binding affinity of herbicide with the ALS and disrupt time-dependent cumulative inhibition [28].

Herbicide metabolism, mediated mainly by Cyt-P450 or GSTs, is the most challenging NTS mechanism, as it is capable of conferring cross and/or multiple resistance/tolerance to herbicides [29]. ALS inhibitors tend to be metabolized slowly, which allows them to interact with the ALS enzyme longer, but, at the same time, these herbicides are slow-acting, promoting metabolic detoxification [28,30]. Cyt-P450s and GSTs are superfamilies of detoxification enzymes that may produce various chemical reactions that reduce the phytotoxic potential of herbicides, allowing tolerant/resistant plants to survive [20]. Malathion, PBO, and NBD-Cl are potent inhibitors of Cyt-P450 or GST [31], reversing tolerance or resistance to herbicides, i.e., plants treated with these substances may become sensitive to herbicides. However, these substances can affect the germination or growth of plants [32,33]. This response depends on each species, and the growth of *F. pyramidata* was not affected by the treatment of malathion, PBO, and NBD-Cl. Cyt-P450s did not metabolize flazasulfuron; however, the efficacy of this herbicide, combined with NBD-Cl, improved, i.e., herbicide tolerance was reversed rendering the *F. pyramidata* plants sensitive to flazasulfuron. This suggest that the rapid GST-mediated metabolism of flazasulfuron was the main mechanism of tolerance in *F. pyramidata* GSTs act in phase II of plant metabolism, adding conjugates and sugars to the herbicide molecule. This can occur without the herbicides being activated in phase I [20]. GSTs have been reported to be responsible for the metabolic degradation of several herbicides, such as atrazine (PSII inhibitor) [34], trifluralin (microtubule inhibitor) [32], mesosulfuron-methyl (ALS inhibitor) [35], S-metolachlor (very-long-chain fatty acid inhibitor) [36], among others.

ALS inhibitors are a fundamental tool for Spanish olive growers to control weeds; however, in olive groves infested with *F. pyramidata,* its use is not recommended, since this species presented cross-tolerance to most of the ALS-inhibiting herbicides. In addition, a low frequency of multiple tolerance to 2,4-D and fluroxypyr was detected; therefore, synthetic auxins cannot be relied on to control long-term tolerant weeds, as observed in *C. acanthoides* from Argentina [27]. In contrast, there is still a great diversity of herbicides with different mechanisms of action that present excellent levels of control of *F. pyramidata*, including some mixtures of ALS inhibitors and synthetic auxins with glyphosate.

5. Conclusions

Filago pyramidata, a new weed invading Spanish olive groves from southern Spain, presented a high level of natural tolerance to flazasulfuron, with LD_{50} values hiher than the field dose of this herbicide, i.e., 50 g ai ha^{-1} of flazasulfuron controlled less than 50% of the individuals of this species in the field. In addition, *F. pyramidata* exhibited moderate to high cross-tolerance to most ALS-inhibiting herbicides, except to the triazolopyrimidine florasulam. Tolerance to flazasulfuron was conferred mainly by GSTs acting in phase II of plant metabolism. It seems that this enzymatic complex also participates in the tolerance to synthetic auxins exhibited by *F. pyramidata* plants. Although the level of tolerance to 2,4-D and fluroxypyr was low, herbicides with this mechanism of action cannot be trusted for the control of *F. pyramidata* in the long-term. On the other hand, herbicides inhibitors of the cellulose synthesis, EPSPS, HPPD, PPO, PSI, and PSII, and some mixtures of ALS inhibitors and synthetic auxins with glyphosate, are excellent alternatives for the control of *F. pyramidata.*

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