Environmental Pollution 322 (2023) 121140

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Enhanced detoxification via Cyt-P450 governs cross-tolerance to ALS-inhibiting herbicides in weed species of *Centaurea*☆

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ARTICLE INFO

Keywords:
Acetolactate synthase
Cornflower
Cyt-P450
Maltese star-thistle
North african knapweed
Tribenuron-methyl

ABSTRACT

Centaurea is a genus of winter weeds with a similar life cycle and competitive traits, which occurs in small-grains production fields in the central-southern of the Iberian Peninsula. However, most of herbicides recommended for weed management in wheat show poor control of *Centaurea* species. This study summarizes the biology, herbicide tolerance to acetolactate synthase (ALS) inhibitors, and recommended chemical alternatives for the control of *Centaurea* species. Four species (*C. cyanus* L., *C. diluta* Aiton, *C. melitensis* L. and *C. pullata* L. subsp. *baetica* Talavera), taxonomically characterized, were found as the main important broadleaf weeds in small-grains production fields of the Iberian Peninsula. These species showed innate tolerance to tribenuron-methyl (TM), showing LD_{50} values (mortality of 50% of a population) higher than the field dose of TM (20 g ai ha^{-1}). The order of tolerance was *C. diluta* (LD_{50} = 702 g ha^{-1}) ≫ *C. pullata* (LD_{50} = 180 g ha^{-1}) ≫ *C. cyanus* (LD_{50} = 65 g ha^{-1}) > *C. melitensis* (LD_{50} = 32 g ha^{-1}). *Centaurea cyanus* and *C. melitensis* presented higher foliar retention (150–180 μL herbicide solution), absorption (14–28%) and subsequent translocation (7–12%) of TM with respect to the other two species. *Centaurea* spp. plants were able to metabolize ^{14}C-TM into non-toxic forms (hydroxylated OH-metsulfuron-methyl and conjugated-metsulfuron-methyl), with cytochrome P450 (Cyt-P450) monooxygenases being responsible for herbicide detoxification. *Centaurea cyanus* and *C. mellitensis* metabolized up to 25% of TM, while *C. diluta* and *C. pullata* metabolized more than 50% of the herbicide. *Centaurea* species showed 80–100% survival when treated with of florasulam, imazamox and/or metsulfuron-methyl, i.e., these weeds present cross-tolerance to ALS inhibitors. In contrast, auxin mimics herbicides (2,4-D, clopyralid, dicamba, fluroxypir and MCPA) efficiently controlled the four *Centaurea* species. In addition, the mixture of ALS-inhibitors and auxin mimics also proved to be an interesting alternative for the control of *Centaurea*. These results show that plants of the genus *Centaurea* found in the winter cereal fields of the Iberian Peninsula have an innate tolerance to TM and cross-resistance to other ALS-inhibiting herbicides, governed by reduced absorption and translocation, but mainly by the metabolization of the herbicide via Cyt-P450.

1. Introduction

Wheat is one of the main crops worldwide, both as a food staple and for the cultivated area designated to the crop. In addition, it is one of the crops in which more research has been carried out on herbicide-based weed control (Domínguez-Mendez et al., 2019; Vázquez-García et al.,

☆ This paper has been recommended for acceptance by Parvaiz Ahmad.
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https://doi.org/10.1016/j.envpol.2023.121140
Received 14 September 2022; Received in revised form 31 December 2022; Accepted 21 January 2023
Available online 24 January 2023

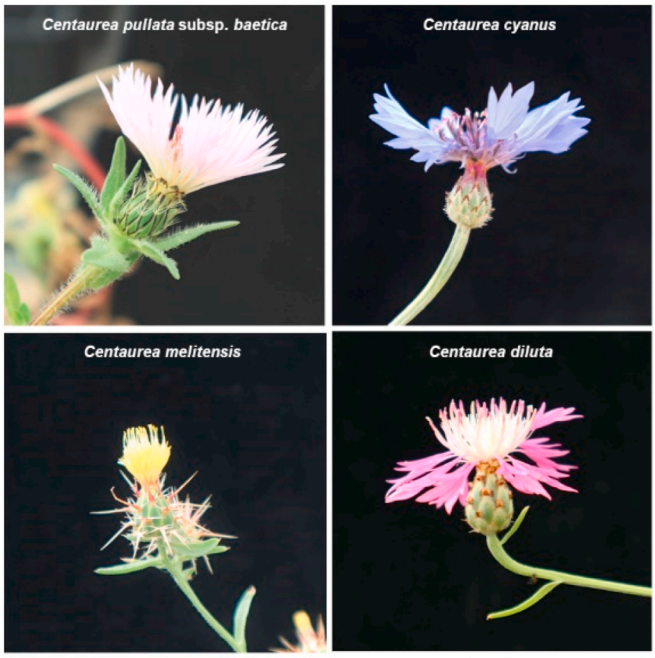

Fig. 1. Seed heads (capitula) of *Centaurea pullata* subsp. *baetica*, *Centaurea cyanus*, *Centaurea mellitensis* and *Centaurea diluta* showing the distinctive involucral bracts.

2022). As a result, a wide variety of effective herbicides are now available to farmers for the control of a wide range of weeds. Paradoxically, after more than half a century of effective chemical control, weed problems have not disappeared and in many cases have increased (Heap, 2022). Different species in the genus *Avena, Centaurea, Lolium,* or *Phalaris* are among the most troublesome wheat weeds in the southern half of Iberian Peninsula with a Mediterranean-climate (Alentejo in Portugal and Andalusia in Spain) (Saavedra et al., 2018; Torra et al., 2022).

The genus *Centaurea* L., widely distributed and diversified in the Iberian Peninsula, comprises 94 species, predominantly perennial (Devesa, 2014), but also there are some annual species (13 species) that occur mainly on the verges of the roads and field margins. However, *C. cyanus* L., *C. diluta* Aiton, *C. melitensis* L. and *C. pullata* subsp. *baetica* Talavera (Fig. 1) occur in annual and perennial crops of the Iberian Peninsula (Saavedra et al., 2018). *Centaurea cyanus* is native to the central-eastern Mediterranean that occurs in cereal crops in temperate zones around the world. The other three *Centaurea* species are native of the arid regions of the western Mediterranean (IPNI, 2022). These Asteraceae species are winter annual weeds showing staggered emergence from early fall rains to late spring, overwintering as rosettes in grain-producing areas (Stankiewicz-Kosyl et al., 2020), which have recently gained relevance in winter cereal fields of the Iberian Peninsula (Torra et al., 2022). Among the four species, *C. diluta* seems to be the most frequent, since seeds of this species have been found in up to 21% of the cereal fields in Western Andalusia (Domínguez-Borrero et al., 2015). Information on the proper management of *Centaurea* species is scarce. In addition, there are suspicions that *C. cyanus* L., *C. diluta, C. melitensis* and *C. pullata* are tolerant to many herbicides currently authorized in Europe for weed control in cereal-producing areas, especially inhibitors of acetolactate synthase (ALS; EC 2.2.1.6).

ALS is a key enzyme catalyzing the synthesis of branched-chain amino acids (BCAAs) in plants and microorganisms (Xu et al., 2021a, b). There are six chemical classes of commercial herbicides that inhibit ALS, including sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-benozoatess (PTB), triazolinones (T) and sulfonanilides (SUL), being the IMI and SU the largest, most important and used (Rosario et al., 2011; Rey-Caballero et al., 2017), which are among the most important herbicides for weed control in cereal fields (Rosario et al., 2011). Otherwise, herbicide tolerance is "the inherent ability of a species to survive and reproduce after herbicide treatment at field rates", i.e., they are plants that do not subjected to selection pressure and yet are capable of surviving an herbicide treatment. Herbicide tolerance could involve non-target site (NTS) and target-site (TS) mechanisms. NTS can be caused by limited absorption, translocation, sequestration in vacuole and by herbicide metabolism (Yu and Powles, 2014; Yang et al., 2016). In contrast, the TS can be caused by mutations and ALS overexpression levels (Lonhienne et al., 2022; Palma-Bautista et al., 2021).

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

Herbicide	Company	Commercial Product	MOA (HRAC)	Field dose (g ai ha^{-1})
Tribenuron-methyl (TM)	Nufarm	Primma® SL	2 (B)	20
Florasulam	Nufarm	Prosulam® SL	2 (B)	5
Imazamox	BASF	Pulsar® 40 SC	2 (B)	40
Metsulfuron	Nufarm	Isomexx® SL	2 (B)	4
2,4-D	Nufarm	U 46 D Complet® SL	4 (O)	600
Clopyralid	Corteva	Lontrel® SG	4 (O)	100
Dicamba	Syngenta	Banvel® D SL	4 (O)	150
Fluroxypir	Nufarm	Praxis® EC	4 (O)	200
MCPA	Nufarm	U 46 SP Fluid® SL (U-46)	4 (O)	1000
TM + MCPA	Nufarm	Primma® SL + U-46	2 + 4	5 + 1000
Floransulam + MCPA	Nufarm	Prosulam® SL + U-46	2 + 4	20 + 1000
Imazamox + MCPA	BASF + Nufarm	Pulsar® 40 SC + U-46	2 + 4	40 + 1000
Metsulfuron + MCPA	Nufarm	Isomexx® SL + U-46	2 + 4	4 + 1000

Since 2018, cereal and legume farmers in the center-south of the Iberian Peninsula have observed low efficacy of ALS-inhibiting herbicides on species of the genus *Centaurea*, particularly *C. diluta* in Andalusia, Spain and *C. pullata* in Alentejo, Portugal. In these areas, the application of tribenuron-methyl (TM) on wheat and imazamox on oilseed rape and/or Clearfield sunflower without rotation with herbicides of other mode of action is a common practice among farmers. The main objective of this study was to determine the tolerance level to ALS-inhibiting herbicides of four different *Centaurea* species (*C. cyanus*, *C. diluta*, *C. melitensis* and *C. pullata*), collected in cereal areas of the southern half of the Iberian Peninsula, where these herbicides have been never applied, and to characterize the NTS mechanisms (foliar retention, absorption, translocation and metabolism) that could probably endow tolerance to TM, and finally look for alternative chemical control options of these species.

2. Material and methods

2.1. Chemical

Table 1 lists the trade herbicides used in this study to determine the physiological effects on the studied populations of *Centaurea* spp. Tribenuron-methyl radiolabeled with ^{14}C (^{14}C-TM) was used to perform absorption, translocation and metabolism assays. This herbicide together with its metabolites (MM, metsulfuron-methyl; OH-MM, hydroxylated metsulfuron-methyl) were supplied by Dupont de Nemours & Co., Nambsheim, France).

2.2. Collection of centaurea seeds

Seeds from mature *Centaurea* spp. were collected in places without record of previous herbicide application from different localities of the

Table 2
Location in the Iberian Peninsula of the sampled seed source populations of *Centaurea* spp.

Species	Country	City	Coordinates
C. cyanus	Spain	Ciudad Real	38°31′04.1″N 3°10′00.8″W
C. diluta	Spain	Cordoba	37°42′43.2″N 4°37′42.9″W
C. melitensis	Spain	Sevilla	37°15′ 39″ N 6°59′33″W
C. pullata	Portugal	Beja	38° 02′ 06″ N 7° 53′ 26″ W

center and South of the Iberian Peninsula. The details of harvest localities are shown in Table 2. The plant capitula were collected from around five mature plants that were randomly distributed over the area. The samples were stored in paper envelopes and placed at 4 °C for two weeks. Then, the achenes were obtained by manually scarification in laboratory. Seeds were germinated in Petri dishes with filter paper moistened with distilled water and placed in a growth chamber at 28/18 °C (day/night) with a photoperiod of 16 h, 350 μmol m^{-2} s^{-1} photosynthetic photon flux, and 80% relative humidity. Seedlings were transplanted into 250-mL pots containing sand/peat (1:2, v/v) and placed in a greenhouse at 28/18 °C (day/night) with a 16 h photoperiod.

2.3. Dose–Response curves

Commercial TM was applied to four *Centaurea* plants at the four-leaf stage using a bench-type track sprayer (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 300 L ha^{-1} at 250 kPa at a height of 50 cm. The TM doses tested were: 0, 2.5, 5, 10, 25, 50, 100 and 200 g ai ha^{-1} for *C. cyanus, C. melitensis* and *C. pullata* and 0, 25, 50, 100, 200, 400, 800 and 1200 g ai ha^{-1} for *C. diluta*. Five plants of each population, chosen randomly, were treated per herbicide dose. After treatments, plants were taken to a greenhouse, held under a temperature regime of 28/18 °C day/night, and watered as necessary. Four weeks after application (WAA), the number of dead plants was recorded. Subsequently, plants were cut at ground level and weighed. The data of fresh weight and plant mortality were transformed into a percentage relative to the untreated control to estimate the LD_{50} (herbicide dose required to kill by 50% a weed population) and GR_{50} (dose required to reduce shoot weight by 50% relative to the control) values.

2.4. TM foliar retention

The amount of TM retained in leaves was determined on plants at BBCH-14 stage of *Centaurea* species were sprayed with 20 g ai ha^{-1} in 300 L ha^{-1} for TM plus 100 mg L^{-1} Na-fluorescein using the same equipment and calibration described below, following the methodology described by Palma-Bautista et al. (2021). Two hours after treatment (HAT) *Centaurea* plants were cut at ground level and the tissue was submerged in test tubes with 50 mL of 5 mM NaOH for 30 s to remove the spray solution. The washing solution was recovered in glass flasks. Subsequently, plants were placed on filter paper, oven-dried at 80 °C for 48 h and weighed. Fluorescein absorbance was determined using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with an excitation wavelength of 490 nm and an absorbance wavelength at 510 nm. Five repetitions were used for each treatment in a completely randomised design. The experiment was repeated twice and the results as μL of sprayed solution retained per g dry weight were combined for statistical analysis.

2.5. ALS activity essay

Three grams of young leaf tissue were taken from each population of *Centaurea* spp. and immediately frozen in liquid N_2 until use (Hatami et al., 2016). ALS was extracted from *Centaurea* plants for assays with technical grade TM at different concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μM). Total protein content was measured using the Bradford method (Bradford, 1976). The maximum specific activity of ALS (nmol acetoin per mg protein per hour) was measured in the absence of herbicide with three technical replicates per *Centaurea* species. The concentration of TM required to reduce ALS activity by 50% (I_{50}) was determined according to Cruz-Hipolito et al. (2009).

2.6. ^{14}C-TM absorption and translocation

Centaurea plants were treated on the adaxial surface of the second leaf with ^{14}C-TM solutions mixed with TM commercial formulation (Palma-Bautista et al., 2021), using a micro-applicator PB-600 (Hamilton Company, Reno NV, USA). The final herbicide application rate was 20 g ai ha^{-1} in 300 L ha^{-1} for TM and the specific activity of the solution was 0.834 kBq μL^{-1}. Plants were maintained in the growth chamber under the growing conditions described above until evaluation. At 96 h after treatment (HAT), five plants of each *Centaurea* population were washed (the treated leaf) three times with 1 mL of distilled-water/acetone (1:1, v/v) to recover the unabsorbed ^{14}C-herbicide. The washing solution of each wash was recovered in 5 mL vials of liquid scintillation, where 2 mL of liquid scintillation cocktail (Ultima Gold, PerkinElmer, Netherlands, MA) was subsequently added. Radioactivity of the non-absorbed ^{14}C-TM was analyzed for 10 min by liquid scintillation spectrometry (LSS).

After washing, the plants were removed from the pot carefully and the roots were washed with distilled water. Plants were sectioned into the treated leaf (TL), the remaining shoot tissue (ST) and the root system (RS). Plant tissue portions were stocked in combustion cones, dried at 60 °C for 96 h and combusted for 3 min in a Packard Tri Carb 307 biological sample oxidizer (Packard Instruments, Meriden, CT, USA). Released $^{14}CO_2$ was trapped and counted by LSS in an 18 mL mixture of Carbo-Sorb E and Permafluor E+ (1:1 v/v) (PerkinElmer, Packard Bioscience BV). Radioactivity of the combustions was also analyzed by LSS for 10 min. Radioactive values were used to calculate the rates of recovery, absorption and translocation of both ^{14}C-herbicides with formulas proposes by Alcántara-de la Cruz et al. (2021), respectively.

2.7. Metabolism inhibitors effects on resistance to TM

The effect of malathion (M), pyperonylbutoxide (PBO) and 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) on the level of tolerance to TM in *Centaurea* spp. plants at the four-leaf stage treated with the field dose of the herbicide (20 g ai ha-^{1}) (Table 1). Prior to herbicide application, 10 plants per species were treated with M, PBO (1000 g ha^{-1}) or NBD-Cl (240 g ha^{-1}) using a laboratory chamber sprayer as before. NBD-Cl was applied 48 h before the TM treatment, while M and PBO were applied only 1 h before. A parallel test was carried out only with the inhibitors to find out if they have any effect on the growth of the different *Centaurea* populations studied. The plants were taken to the greenhouse and kept under the conditions described above until their evaluation. At 4 WAA, the fresh weight of the plants was determined and compared with the TM test without the application of metabolism inhibitors. The experiments were conducted twice at different times.

2.8. TM metabolism

Tribenuron-methyl metabolism was determined according to Palma-Bautista et al. (2021). *Centaurea* plants tolerant to TM were treated (0.834 kBq μL^{-1} $plant^{-1}$) and harvested at 96 HAT following the same methodology as that used for ^{14}C-TM absorption and translocation. The ^{14}C-TM and its metabolites were separated by thin-layer chromatography on a 20 by 20 cm silica gel plate (silica gel 60, Merck, Darmstadt, Germany) with isopropanol, ethyl acetate, ammonia, and distilled water (10:6:3:1 v/v/v/v). The radioactive zones were detected by scanner to obtain radiochromatograms. The radioactivity of each product was quantified with linear analyzer plate equipment (Berthold LB 2821,

Table 3
Parameters of the log-logistic equations used to calculate the TM rates (g ia ha^{-1}) required for 50% reduction of plant fresh weight (GR_{50}) and survival (LD_{50}) of the four *Centaurea* species.

Specie	GR_{50}	95% CI		LD_{50}	95% CI	
		Lower	Upper		Lower	Upper
C. cyanus	**9.6**	7.4	11.8	**64.6**	56.6	72.6
C. diluta	**146.5**	130.8	163.2	**702.3**	528.9	875.7
C. melitensis	**7.8**	6.4	9.5	**31.6**	22.7	40.3
C. pullata	**23.3**	16.3	30.9	**179.9**	149.8	202.4

Wildbald, Germany), while the separate products were determined by comparison with standards (TM, metsulfuron-methyl (MM), metsulfuron-methyl hydroxylated (MM-OH) and conjugate-MM). The experiment was performed with three plants, each with three technical replicates.

2.9. Alternatives herbicides to control Centaurea spp.

A greenhouse screening test was carried out to ascertain whether these *Centaurea* populations can survive the application of ALS-inhibitors and auxin mimic herbicides used in cereal crop in Southern, Spain. Herbicides treatments listed in Table 1 were applied in post-emergence on 4-leaf *Centaurea* plants. The experiments were arranged in a completely randomized design using 10 plants (one plant per pot) per herbicide-treatment in a treatment chamber as has been exposed above. At 4 WAA the fresh weight was determined, and the number of surviving plants was counted. The experiments were conducted twice at different times (spring and fall), and the average of the two runs was obtained.

2.10. Data analysis

TM rates necessary reducing weight (GR_{50}), survival plant (LD_{50}) and ALS activity (I_{50}) by 50% were estimated by non-linear regression with the three parameters equation: $Y = d/[1+(\log(x)-\log(e))^b]$; where d is the upper limit, b the slope of the curve at the point of inflection halfway between the upper and lower limit (fitted at zero), e is the herbicide dose in GR_{50}, LD_{50} and I_{50}; x ire the increased herbicide doses of the dose-response curves. The regression analyses were performed using the "drc" package (Ritz et al., 2015) in the "R" program (version 4.1.3).

An ANOVA was performed for the data of foliar retention, metabolism inhibitors, metabolism assay and alternative herbicides. When required, Tukey's test at 0.05 probability was used to grouping. AOVA and mean comparison analyses were performed in Statixtix 10 (Analytical Software, USA).

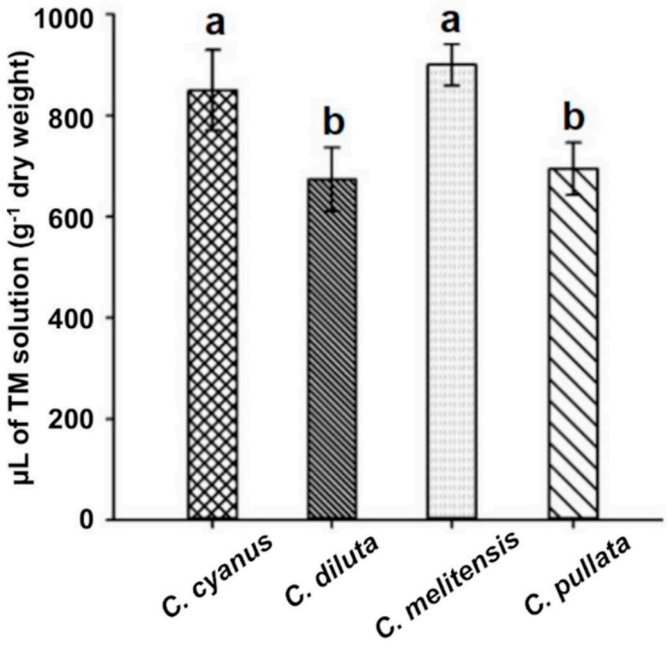

Fig. 2. TM foliar retention in four *Centaurea* species. Same letters are not different by the Tukey's test at 95%. Vertical bars indicate the standard error of the mean (n = 10).

Table 4
Parameters of the log-logistic model used to estimate the concentration of TM necessary to reduce the activity of the ALS enzyme by 50% (I_{50}) in *Centaurea* spp. populations.

Specie	ALS[a] activity	d	b	I_{50} (µM)	*p*-value	TF[b]
C. cyanus	370.3 ± 16.9	97.6	3.6	71.8	<0.001	1.03
C. diluta	379.8 ± 18.0	98.1	3.5	71.1	<0.001	1.02
C. melitensis	372.1 ± 13.3	97.6	3.5	69.7	<0.001	–
C. pullata	375.9 ± 5.1	97.6	3.5	70.1	<0.001	1.00

[a] Nmol of acetoin per mg of protein per hour.
[b] Tolerance factor.

3. Results

3.1. Whole plant assays

Fresh weight of *Centaurea* plants was reduced by 50% with different doses of TM, that ranged from 7.8 to 146.5 g ai ha^{-1}. In all cases, the TM doses required to control the four *Centaurea* populations by 50% (LD_{50}) were higher than the recommended field dose (20 g ai ha^{-1}) used in cereal and annual legume crops in Spain. *Centaurea diluta* was the most tolerant species to the TM treatment. Thus, based on the GR_{50} and LD_{50} values, the tolerance levels to TM in the studied populations were in the order *C. diluta* > *C. pullata* > *C. cyanus* > *C. melitensis* (Table 3).

3.2. Foliar retention of TM

The amount of TM solution retained on leaf differed between *Centaurea* species. *Centaurea cyanus* and *C. mellitensis* retained 150–180 µL more herbicide solution (~875 µL TM g^{-1} dry weight) than *C. diluta* and *C. pullata* (Fig. 2).

3.3. ALS activity essay

In the absence of TM, ALS-specific activity levels (nmol acetoin per mg protein per hour) in the four *Centaurea* populations did not differ. The enzymatic activity of ALS of the different *Centaurea* species was inhibited with similar amounts of TM, i.e., no contrasting results were found in the I_{50} values, which ranged from 370 to 380 µM of TM without differences between populations (Table 4).

Table 5
^{14}C-TM absorption and radiolabel translocation from the treated leaves in the *Centaurea* spp. plants 96 h after treatment with TM at 20 g ai ha^{-1}.

Specie	Absorption (%)	Translocation (% of Absorbed)		
		TL	ST	RS
C. cyanus	79.7 ± 2.3 b	74.6 ± 2.7 bc	12.0 ± 1.0 ab	13.4 ± 1.3 a
C. diluta	59.4 ± 2.4 cd	85.0 ± 2.6 a	9.8 ± 0.7 b	5.2 ± 0.7 c
C. mellitensis	87.5 ± 3.5 a	72.1 ± 1.5 c	14.0 ± 1.2 a	13.9 ± 1.1 a
C. pullata	65.1 ± 3.4 c	81.5 ± 2.9 ab	9.4 ± 0.8 b	9.1 ± 0.6 b

Same letter within a column are not different by the Tukey test at 95%. ± Standard error of the mean (n = 5).

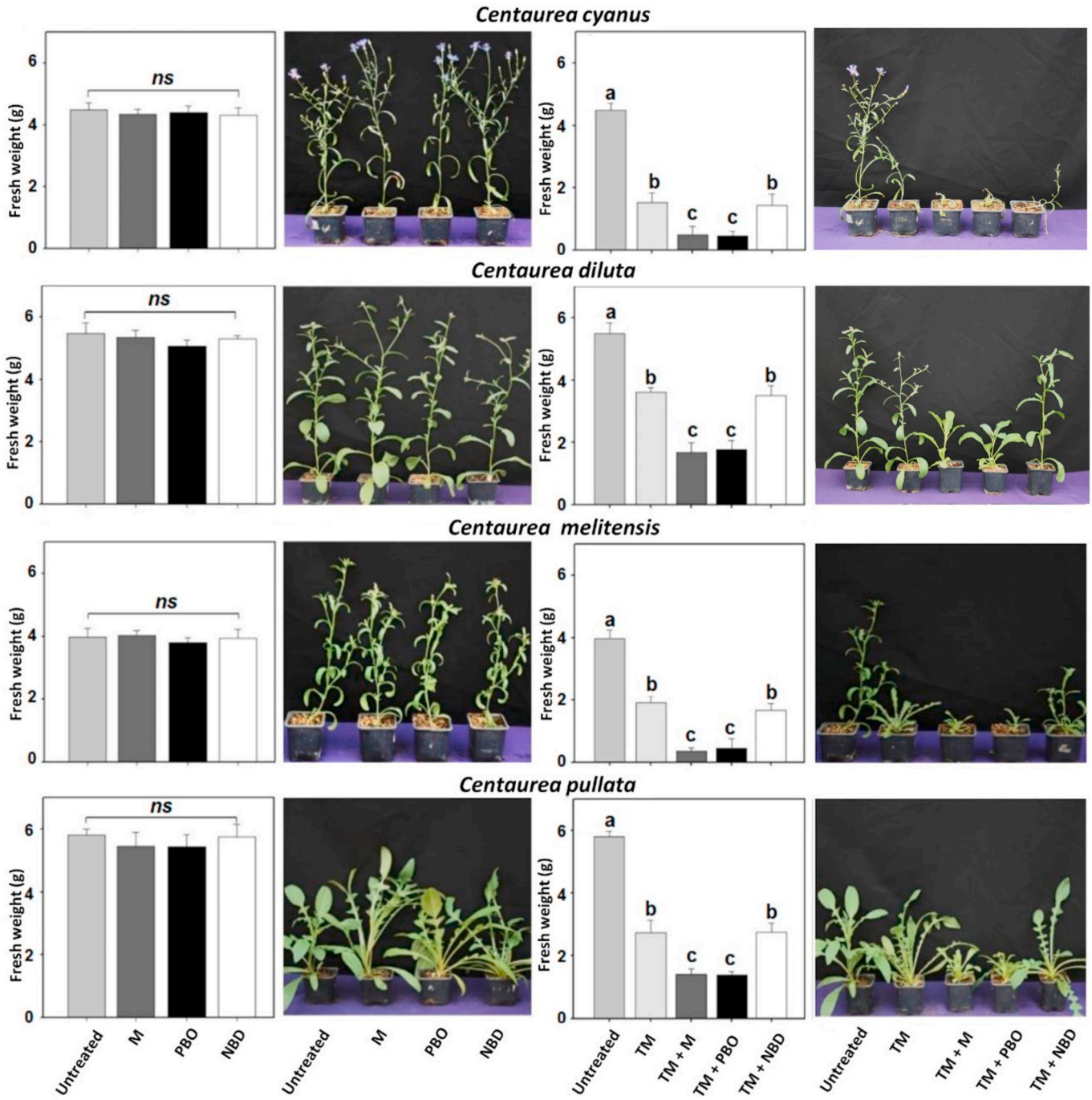

Fig. 3. Response of *Centaurea* spp. to tribenuron-methyl presence or absence Cyt P450 and GST inhibitors. TM – Tribenuron-methyl; M – malathion; PBO – piperonyl butoxide; NBD-CL – 4-Chloro-7-nitro-2,1,3-benzoxadiazole. Same letters are not different by the Tukey test at 95%. Vertical bars indicate the standard error of the mean (n = 20).

3.4. ^{14}C-TM absorption and translocation

The absorption and translocation patterns of ^{14}C-TM differed between *Centaurea* species. Plants of *C. pullata* and *C. diluta* absorbed between 59 and 65% of the ^{14}C-TM applied at 96 HAT. The species retained more than 80% of the ^{14}C-herbicide in the TL, and only translocated ~9.6% to the ST and from 5 to 9% to the RS. On the other hand, *C. melitensis* and *C. cyanus* plants absorbed 20–25% more ^{14}C-TM than *C. pulluta* and *C. diluta*, resulting in a higher translocation of ^{14}C-herbicide from the TL to the ST (3–5%) and the RS (5–9%) (Table 5).

3.5. Metabolism inhibitors effects on resistance to TM

M, PBO, and NBD-CL, applied alone, had no phytotoxic effects on *Centaurea* plants, showing similar growth that the untreated control. TM alone or in combination with NBD-CL (glutathione S-transferases (GST) inhibitor) caused a similar growth reduction in *Centaurea* plants, ranging from 35 to 60% depending on the species. However, the combination of the cytochrome P450 monooxygenase (Cyt-P450s) inhibitors, M and PBO, with TM reduced the growth by 70–92%, with *C. cyanus* and *C. mellitensis* being the species most susceptible to these combinations (Fig. 3).

Table 6
TM and its metabolites (expressed as %) in *C. cyanus, C. diluta, C. melitensis* and *C. pullata* plants treated with 20 g ai ha^{-1} and harvested 96 HAT.

Specie	Herbicide[a]	Metabolites[a] (percentage)		
	TM	MM	OH-MM	Conjugate-MM
C. cyanus	73.2 ± 2.1 a	16.9 ± 1.7 a	5.2 ± 0.5 c	4.7 ± 0.5 c
C. diluta	35.7 ± 2.2 c	12.7 ± 1.1 b	16.4 ± 0.7 a	35.2 ± 0.9 a
C. melitensis	75.1 ± 3.0 a	15.3 ± 0.5 a	5.5 ± 0.5 c	4.1 ± 0.7 c
C. pullata	50.1 ± 2.5 b	13.3 ± 1.2 b	9.3 ± 0.9 b	27.3 ± 0.8 b

[a] Mean values±standard errors are given; TM – tribenuron-methyl; MM – metsulfuron-methyl; OH-MM – OH-hydroxylated metsulfuron-methyl; Conjugate-MM – conjugate-metsulfuron-methyl. Same letter within a column are not different by the Tukey test at 95%. ± Standard error of the mean (n = 9).

3.6. TM metabolism

All *Centaurea* species were able to metabolize ^{14}C-TM into MM, OH-MM and/or conjugate-MM, but this metabolization was different for each species. *Centaurea diluta* and *C. polluta* metabolized 65 and 50% of the ^{14}C-herbicide, respectively, while *C. cyanus* and *C. melitensis* only 25–27%. The amount of ^{14}C-TM transformed into MM (toxic form) was similar between *Centaurea* species (12.7–16.9%); however, each species metabolized different amounts of ^{14}C-herbicide in non-toxic forms (OH-MM and conjugated-MM). *Centaurea cyanus* and *C. melitensis* metabolized less than 10% of ^{14}C-TM into non-toxic forms, while C. *polluta* transformed up to 36.6%. The species that transformed the most ^{14}C-TM into OH-MM and conjugate-MM (14.6 and 35.2%, respectively) was *C. diluta* (Table 6).

3.7. Alternative herbicides to control Centaurea spp.

Centaurea species showed different susceptibility levels to the ALS-inhibiting herbicides tested. *Centurea cyanus* and *C. melitensis* showed the greatest fresh weight reductions; however, in most treatments there was a high percentage of plant survival. For example, imazamox reduced the fresh weight of *C. melitensis* by 84%, but 100% survived the treatment. On the other hand, TM and MM reduced the fresh weight by up to 95%, causing mortality of 20 and 60% of the plants. The fresh weight reduction reached up to 50% in *C. diluta* and *C. pullata* with some treatments, but 100% of the plants of both species survived to the treatmetns of TM, floransulam, imazamox and MM. In contrast, treatments that included synthetic auxins (2,4-D, clopyralyd, flroxipyr, or MCPA), applied alone or in combination with an ALS-inhibitor, reduced fresh weight and caused plant mortality by 100% of the *Centaurea* species (Table 7).

4. Discussion

ALS-inhibiting herbicides play a crucial role in controlling weeds in winter cereals and a variety of Clearfield crops (Alcántara-de la Cruz et al., 2021; Rey-Caballero et al., 2017; Vázquez-García et al., 2022). However, the present study revealed that the *Centaurea* species studied possess a high tolerance to ALS-inhibiting herbicides, which has hindered their control, making them increasingly relevant weeds in the southern winter grain-producing areas of the Iberian Peninsula (Saavedra, 1997; Stankiewicz-Kosyl et al., 2021). The estimated LD_{50} values for the *Centaurea* species studied were higher than the field dose (20 g ai ha^{-1}) of TM used in the Iberian Peninsula, manifesting an innate tolerance to ALS-inhibiting herbicides. The estimated GR_{50} values for the *Centaurea* species ranged between 7.8 (*C. melitensis*) and 146.5 (*C. diluta*) g ai ha^{-1}, i.e., the level of tolerance to ALS inhibitors between *Centaurea* species is variable. The presence of different tolerance mechanisms or, alternatively, the high prevalence of one of them could be implied. Worldwide, the only species studied has been *C. cyanus,* one of the main weeds found among winter crops in Poland (Wrzesinska and Praczyk, 2021). The authors found GR_{50} values between 4.3 and 6.1 g ai ha^{-1}, which were lower compared to those estimated in this study.

4.1. Unravelling tolerance mechanisms

Foliar retention of TM solution was lower in *C. cyanus and C. melitensis* than in *C. pullata* and *C. diluta*, allowing a greater penetration of the herbicide inside the plant cells of the two former species. The low foliar retention of the herbicide solution may be a mechanism that affected the effectiveness of the TM, which initially explains the differential tolerance between *Centaurea* species to this herbicide, since it reduced the amount of active ingredient that entered the plant (Palma-Bautista et al., 2020; Yanniccari et al., 2021).

^{14}C-TM uptake and translocation patterns differed among the four *Centaurea* species, being lower in *C. diuta* and *C. pullata* compared with *C. cyanus* and *C. melitensis*. These NTS mechanisms have rarely been found to be involved in resistance/tolerance to ALS-inhibiting herbicides. In other dicotyledonous species such as *Sinapis alba, S. arvensis* and *Papaver rhoeas*, common in the cereal-producing areas of the Iberian Peninsula, showed no differences in ^{14}C-TM uptake or translocation rates between resistant *vs.* susceptible biotypes (Rosario et al., 2011; Rey-Caballero et al., 2017; Gherekhloo et al., 2018; Alcantara de la Cruz., 2021). Therefore, the low uptake and translocation of ^{14}C-TM observed could by itself explain the high innate tolerance in *C. diuta* and *C. pullata* and medium tolerance in *C. cyanus* and *C. melitensis*. However, these NTS mechanisms alone hardly confer tolerance to field doses of TM, therefore, probably other NTS mechanisms, such as metabolism,

Table 7
Effect of the alternative herbicide treatments tested at field doses in greenhouse on plant fresh weight (fw) and survival (%) at 28 DAT for the studied *Centaurea* populations.

Herbicide[a]	*C. cyanus*		*C. diluta*		*C. melitensis*		*C. pullata*	
	Fw	%Survival	Fw	%Survival	Fw	%Survival	Fw	%Survival
Control	4.5 ± 1.6	100	5.5 ± 0.6	100	3.6 ± 0.9	100	5.2 ± 0.3	100
TM	1.3 ± 0.3	100	5.0 ± 1.5	100	0.3 ± 0.3	80	2.9 ± 1.0	100
Florasulam	1.0 ± 0.7	100	4.3 ± 0.4	100	2.1 ± 0.8	100	3.1 ± 0.9	100
Imazamox	1.1 ± 0.3	100	4.1 ± 1.3	100	0.6 ± 0.2	100	2.7 ± 0.4	100
MM	2.3 ± 1.0	100	3.9 ± 1.1	100	0.2 ± 0.1	40	3.3 ± 0.7	100
2,4-D	0	0	0	0	0	0	0	0
Clopyralid	0	0	0	0	0	0	0	0
Dicamba	0	0	0	0	0	0	0	0
Fluroxypir	0	0	0	0	0	0	0	0
MCPA	0	0	0	0	0	0	0	0
Florasulam + MCPA	0	0	0	0	0	0	0	0
Tribenuron-methyl + MCPA	0	0	0	0	0	0	0	0
Imazamox + MCPA	0	0	0	0	0	0	0	0
MM + MCPA	0	0	0	0	0	0	0	0

[a] N = 10 plants (1pl/pot). ± Standard error of the mean (n = 20).

also are involved in the TM tolerance shown by *Centaurea* species.

The application of Cyt-P450s and GSTs inhibitors alone did not affect the growth of *Centaurea* spp. However, when applied in combination with TM, the Cyt-P450s inhibitors (M and PBO) reversed the observed tolerance to this herbicide, while the GST inhibitor (NBD-Cl) did not reduce the phytotoxic effects of TM. Cyt-P450 monooxygenases add a functional group to the herbicide molecule by oxidation, reduction or hydrolysis in the phase I of plant metabolism (Gaines et al., 2020). GSTs enzymes act in the phase II of metabolism, where the herbicide undergoes more complex changes due to the addition of conjugates and sugars, being able of directly detoxify some herbicides without depending on phase I activation (Gaines et al., 2020). With these chemical reactions, the herbicide loses its phytotoxic potential and tolerant/resistant plants survive the treatment; therefore, the inhibition of these plant metabolic enzyme complexes reveals their participation in tolerance/resistance to herbicides. Both M and PBO as Cyt-P450s inhibitors are capable of reversing resistance to several herbicides such as mesosulfuron-methyl (ALS-inhibitor), fenoxaprop-p-ethyl (Acetyl CoA carboxylase-inhibitor), or tembotrione (hydroxyphenylpyruvate dioxygenase-inhibitor), among others (Oliveira et al., 2017; Zhao et al., 2018; Wu et al., 2022). GSTs have been also found participating in the metabolic degradation of various herbicides such as triazines (photosystem II inhibitor), sulfonylureas (ALS inhibitors), aryloxyphenoxypropionates (ACCase inhibitors), among others (Cummins et al., 2011). However, our results suggest that the enzyme complex responsible for the metabolism of TM into less toxic compounds was only the Cyt-P450.

^{14}C-TM metabolism studies showed that all four *Centaurea* species are capable of metabolizing the herbicide in non-toxic forms (hydroxylated OH-metsulfuron-methyl and conjugated metsulfuron-methyl). These species showed different metabolic capacities, which are consistent with the previously exposed parameters that measure the responses of the population and plants to the application of herbicides. That is, the species with the highest capacity to metabolize TM (*C. diluta* and *C. polluta*) also showed the highest levels of tolerance observed in the dose-response and absorption and translocation assays, and vice versa. Herbicide metabolism is a complex process involving a variety of metabolic detoxification enzymes, including P450, GST, glycosyltransferases (GT), ATP-binding cassette (ABC) transporters, aldo-keto reductase (AKR), oxidases, esterases, hydrolases and peroxidases (Gaines et al., 2020). These results corroborate the results of the previous experiment with inhibitors of Cyt-P450s and GSTs, confirming that only Cyt-P450 monooxygenases participates in the metabolic detoxification of TM in *Centaurea* species. The increased tolerance to tembotrione in *Amaranthus tuberculatus* from Nebraska was also due to enhanced P450 activity (Owen et al., 2012). Other studies show that high bensulfuron-methyl resistance in *Sagittaria trifolia* and thifensulfuron-methyl resistance in *Ipomoea purpurea* were due to higher Cyt-P450s activity (Zhao et al., 2017; Xu et al., 2021a,b).

Basal enzyme activity and inhibition rate by 50% (I_{50}) of ALS were similar, suggesting that TS mechanisms were not involved in TM tolerance in *Centaurea* species; however, more studies are needed to confirm or rule out this hypothesis. Differences in basal activity can reveal gene overexpression of the herbicide's target enzyme, while differences in the enzyme inhibition rate by the herbicide are often due to mutations (Gaines et al., 2020; Xu et al., 2021). Although no ALS-inhibiting herbicide molecule mimics the substrates of the enzyme, they inhibit ALS by blocking the channel through which the substrates access the active site (McCourt et al., 2006); therefore, mutations at key positions reduce the binding affinity of herbicides with the ALS (Lonhienne et al., 2022). A recent study revealed four mutations (L179I, N404R, I468V, and V525I) in TM-resistant *C. cyanus* (Wrzesinska and Praczyk, 2021); however, the role of these mutations in conferring resistance to ALS inhibitors has not yet been demonstrated.

4.2. Alternatives to control Centaurea spp.

ALS inhibitors are still efficient in controlling other species and are the basis of weed management in winter crops, therefore, it is difficult for farmers to dispense these herbicides. Regarding this concern, here it was demonstrated that the four *Centaurea* species exhibit different levels of cross-resistance to florasulam, imazamox, metsulfuron and TM, herbicides with different chemical structures within ALS inhibitors. Studies carried out in Spain and Poland with *C. diluta* (Saavedra et al., 2018) and *C. cyanus* (Stankiewicz-Kosyl et al., 2020, 2021) showed that the application of ALS-inhibiting herbicides at field doses resulted in less than 50% control of the populations of these two weeds and, as our work highlights, this is probably due to the high tolerance of *Centaurea* weedy species to these herbicides. However, the treatment with auxin-mimicking herbicides resulted in excellent control of the populations of the four *Centaurea* species tested. Greenhouse results suggest that the mixture of ALS inhibiting herbicides and auxin mimics has proven to be a very effective tool for the control of different species of genus *Centaurea* in cereal crops.

5. Conclusions

The results of this investigation demonstrated that annual plants of the genus *Centaurea* that occur in winter cereal fields in the Iberian Peninsula possess innate tolerance to TM, and cross-resistance to other ALS-inhibiting herbicides, governed by NTS-tolerance mechanisms. The absorption and translocation of the herbicides, but mainly the metabolization of the TM mediated by Cyt-P450 monooxygenases were involved. Future studies should try to identify putative Cyt-P450 genes encoding enzymes that can degrade TM and establish whether a single or several can confer the different levels of TM metabolism and cross-tolerance to ALS inhibitors in weedy *Centaurea* spp.

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Funding

This work was funded by the Asociacion de Agroquimicos y Medioambiente (Spain) and by national funds through the Fundação para a Ciência e Tecnologia, I.P. with the project UIDB/05064/2020 (VALORIZA).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors thank Sergio Oliva Sánchez for his technical assistance.

Joel Torra gratefully acknowledges support from the Ministerio de Ciencia, Innovación y Universidades (Ramón y Cajal grant RYC2018-023866-I), Spain. Foundation for Science and Technology through the project UIDB/05064/2020 (VALORIZA), Portugal.

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