Weed Technology

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Pro197 and Trp574 substitutions in the acetolactate synthase of corn marigold (*Glebionis segetum*) and their impact on competitive ability against barley

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Research Article

Cite this article: Papapanagiotou AP, Spanos T, Zarrougui NE, Livieratos IC, Eleftherohorinos IG (2023) Pro197 and Trp574 substitutions in the acetolactate synthase of corn marigold (*Glebionis segetum*) and their impact on competitive ability against barley. Weed Technol. **37**: 165–173. doi: 10.1017/wet.2023.17

Received: 27 October 2022
Revised: 27 January 2023
Accepted: 26 February 2023
First published online: 13 March 2023

Associate Editor:
R. Joseph Wuerffel, Syngenta

Nomenclature:
Aminopyralid; clopyralid; dicamba; florasulam imazamox; mecoprop-PP; pyroxsulam; tribenuron; tritosulfuron; barley; *Hordeum vulgare* L.; corn marigold; *Glebionis segetum* L. Fourr.

Keywords:
Weed resistance; *ALS* gene sequence; weed–crop competition

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Abstract

Three suspected resistant (R1, R2, and R3) corn marigold populations collected from winter cereal fields located in central Greece were studied to confirm and elucidate the mechanisms of resistance to acetolactate synthase (ALS) inhibitors and their competitive ability against barley. Whole-plant dose–response assays proved that the three suspected R populations were highly cross-resistant to the ALS inhibitors tribenuron, pyroxsulam + florasulam, and imazamox, whereas their control with synthetic auxin plus ALS inhibitors co-formulated mixtures was increased in the order of tritosulfuron + dicamba < florasulam + clopyralid < tribenuron + mecoprop-P < florasulam + aminopyralid. The *ALS* gene sequence revealed a point mutation in 11 plants of the R1, R2, and R3 populations, which resulted in the substitution of Pro-197-Thr or Trp-574-Leu. By contrast, all three sequenced plants of the susceptible (S) population were found with the wild-type allele encoding Pro-197 and Trp-574. This is the first report of ALS-inhibitor resistance in corn marigold. The competition study between barley and four densities of the S, R2, or R3 populations indicated similar biomass rates for all three populations, suggesting lack of association between the competitive ability of the R populations and the target-site resistance mechanism, which was also confirmed by the similar biomass reduction rates of barley grown in competition with S or R populations.

Introduction

Corn marigold is an annual cross-pollinated branched dicot herb with erect to ascending stems. It grows extensively in the Mediterranean, North Africa, Europe, and Asia (Servi 2021). It has a prolonged germination period and reproductive ability that exceeds 3,000 seeds per plant (Howarth and Williams 1972). Corn marigold is locally frequent in arable land and pastures, preferentially infesting winter cereal crops—especially wheat (*Triticum aestivum* L.) and barley—where it forms dense stands (Frost 1982; Howarth and Williams 1972) resulting in significant reduction in crop productivity.

Control of corn marigold in winter cereals relies mainly on acetolactate synthase (ALS) inhibitors applied either alone or in mixtures with synthetic auxins (Hada et al. 2020, 2021, 2022). Unfortunately, ALS-inhibiting herbicides are the most prone to rapid evolution of target site–mediated resistance, which, in most reported cases, is conferred by a single missense mutation in the *ALS* gene (Yu and Powles 2014). The target-site mutations conferring ALS resistance in various weed species have been shown to affect the Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 positions (Murphy and Tranel 2019). The most detected amino acid substitutions conferring ALS resistance are those affecting Pro-197 and Trp-574 (Beckie and Tardif 2012; Yu and Powles 2014). The synthetic auxin herbicides, having a mode of action similar to the endogenous plant hormone indole-3-acetic acid, are also applied in winter cereals either alone or as mixtures with ALS-inhibiting herbicides for the control of broadleaf weeds, helping to slow the appearance and spread of ALS-resistant weed populations (Hada et al. 2022; Ntoanidou et al. 2017, 2019; Rosario et al. 2011).

Although corn marigold is a weed of economic importance (Frost 1982; Howarth and Williams 1972), studies on competition between wheat or barley and corn marigold do not exist in the literature, to our knowledge. Studies on competition between resistant (R) or susceptible (S) populations of this weed species against winter cereal crops are lacking, although they

provide useful information for the management of this weed and on the ecological evolutionary context of its fitness and adaptation (Vila-Aiub et al. 2009). Therefore, testing the R vs S populations in the presence of a crop is a novel aspect of our research.

During the 2019–2020 growing season, some farmers in central Greece reported unsatisfactory control of this weed in their winter cereal crops (wheat and barley) following the application of the ALS inhibitor tribenuron, which has been repeatedly used in this area for at least 10 yr consecutively. Based on this information, the aims of this study were to (1) test the suspected R corn marigold populations for the evolution of resistance to tribenuron and other ALS-inhibiting herbicides, (2) elucidate the underlying mechanism conferring herbicide resistance, (3) evaluate the efficacy of co-formulations of ALS-inhibiting and synthetic auxin herbicides as a complementary or alternative chemical treatment to prevent further buildup of ALS-resistant weed populations, and (4) investigate the competitive ability of one S and two R populations against barley.

Materials and Methods

Plant Material

A roadside survey was conducted during spring and early summer of the 2020 growing season in winter cereal fields located in central Greece (Larisa region), where failure of corn marigold control with the ALS inhibitor tribenuron had been reported. During the survey, three fields with poor control of this weed were marked as suspected R populations, and seeds were collected from surviving corn marigold plants before crop harvest. Mature seeds were collected by hand from 80–90 individual corn marigold plants from each field and pooled together. In addition, seeds were collected from corn marigold plants grown in an uncultivated area close to the wheat field where the R3 population was field-selected, with no history of exposure to herbicide applications, and these seeds were considered as the S population. The collected seeds were transferred to the laboratory, where they were air-dried, threshed, placed in paper bags, and stored at room temperature (18–25 C).

Whole-Plant Dose–Response Assays to ALS Inhibitors

A pot experiment was conducted under greenhouse conditions at the farm of University of Western Macedonia, Florina, Greece, during October 2020 to January 2021. Plants were grown in 10 × 10 × 10-cm plastic pots, filled with a 1:1:1 (v/v/v) mixture of clay loam soil with peat and sand. Each pot was seeded with approximately 20 corn marigold seeds of each of the S or three suspected R corn marigold populations (R1, R2, and R3) and carefully covered with 1-cm depth of the soil mixture. The emerged plants, at the two-leaf stage, were carefully thinned to six plants per pot, and once plants reached the four-leaf growth stage (5–7 cm tall), herbicide treatments were applied. In particular, the plants of the three suspected R populations were treated with the recommended label rate (1×), 2×, 4×, and 8× rates of the ALS-inhibiting herbicides tribenuron, pyroxsulam + florasulam, and imazamox, whereas the S corn marigold population was treated with the same herbicides applied at ×/8, ×/4, ×/2, and 1× rates. Lower than recommended rates of all herbicides were used because this population was considered susceptible. All herbicide rates along with the surfactants added during application are presented in Table 1. Nontreated checks for the S and putative R populations were also included. All herbicide treatments were applied with a propane-pressurized hand-field plot portable sprayer (AZO-Sprayers, Ede, The Netherlands), having a 2.4-m-wide boom fitted with six 8002 flat-fan nozzles (Teejet Spray System Co., Wheaton, IL). Pots were re-randomized each week to achieve uniform growth conditions for all plants. All R and S populations were evaluated in the same run, but the experiments were conducted twice in a randomized complete block design with three replications for each herbicide dose–treatment. The sprayer was calibrated to deliver a water volume of 300 L ha^{-1} at a pressure of 280 kPa. Corn marigold control was assessed by measuring the aboveground fresh weight of plants 5 wk after treatment (WAT). The dead plants were not evaluated.

Whole-Plant Dose–Response Assays to ALS Mixtures with Synthetic Auxin Herbicides

The three suspected R1, R2, and R3 corn marigold populations were evaluated outdoors in a net-protected area for their response to postemergence-applied co-formulations of ALS-inhibiting plus synthetic auxin herbicides, registered for use in small grain cereals in Greece. The experiment was conducted at the farm of University of Western Macedonia, Florina, Greece, during late February to May 2021, following the same procedure described previously for the ALS-inhibiting herbicides. In particular, the plants of the three suspected R populations, at four-leaf growth stage, were treated with the recommended label rate (1×), 2×, 4×, and 8× rates of the co-formulations tribenuron + mecoprop-P, florasulam + aminopyralid, tritosulfuron + dicamba, and florasulam + clopyralid, whereas the S corn marigold population was also treated with the same herbicides applied at ×/8, ×/4, ×/2, and 1× rates (Table 1). The lower than the recommended rates of all herbicides were used because this population was considered susceptible. Nontreated checks for the S and suspected R populations were also included. The application of the tested herbicides was performed by the propane-pressurized hand-field plot portable sprayer (AZO-Sprayers, Ede, The Netherlands) used previously. Pots were re-randomized each week to achieve uniform growth conditions for all plants. The R and S populations were evaluated in different experiments conducted at the same time due to different rates, whereas the experiments were repeated twice for R and S populations using a randomized complete block design with three replications for each herbicide dose–treatment. Corn marigold control was assessed by measuring the aboveground fresh weight of plants 5 WAT. The dead plants were not evaluated.

ALS Gene Sequencing

The *ALS* gene fragment covering potential mutation sites in the suspected R corn marigold populations was amplified, sequenced, and compared. For the amplification of the *ALS* gene, plant material was collected from R1, R2, and R3 populations, grown in four pots per each R population and in eight pots of the S population. The pots containing R1, R2, and R3 plants and four pots of the S plants were treated with the labeled rate of tribenuron, whereas the other four pots of the S population were left untreated. This treatment was carried out to eliminate individual susceptible plants from the R populations and to ensure the susceptibility of the S population. Leaf tissues from surviving R1, R2, and R3 corn marigold plants and from the untreated S plants were harvested, immediately stored at –28 C, and subsequently subjected to DNA extraction. Genomic DNA was isolated from 3 S and 12 putative R plants (4 plants from each R population), using 90–100 mg of young leaf tissue, according to the "DNeasy® Plant Mini Kit" protocol (QIAGEN, Hilden, Germany). The quality and quantity

https://doi.org/10.1017/wet.2023.17 Published online by Cambridge University Press

Table 1. Source of materials for the products used in the whole-plant dose–response experiments against the S and R corn marigold populations. The first four rates of each herbicide were used for the S population, and the last four rates for the R population.

Herbicide	Trade name	Form[a]	Rate	Manufacturer
			g ai ha^{-1}	
Tribenuron[b]	Granstar	SG	1.87	Corteva Agriscience Hellas, Athens, Greece
			3.75	
			7.5	
			15[c]	
			30	
			60	
			120	
Pyroxsulam + florasulam[b]	Broadway	WG	2.35 + 0.46	Corteva Agriscience Hellas, Athens, Greece
			4.7 + 0.92	
			9.4 + 1.84	
			18.8 + 3.68	
			37.6 + 7.36	
			75.2 + 14.72	
			150.4 + 29.44	
Imazamox[b]	Pulsar	SL	6.25	BASF Hellas, Athens, Greece
			12.5	
			25	
			50	
			100	
			200	
			400	
Tribenuron + mecoprop-P[b]	Granstar Combi	SG	1.35 + 100	FMC, Athens, Greece
			2.7 + 200	
			5.4 + 400	
			10.8 + 800	
			21.6 + 1600	
			43.2 + 3200	
			86.4 + 6400	
Florasulam + aminopyralid	Lancelot	WG	1.23 + 0.62	Corteva Agriscience Hellas, Athens, Greece
			2.46 + 1.24	
			4.92 + 2.48	
			9.84 + 4.96	
			19.68 + 9.92	
			39.36 + 19.84	
			78.72 + 39.68	
Tritosulfuron + dicamba	Arrat	WG	6.25 + 12.5	BASF Hellas, Athens, Greece
			12.5 + 25	
			25 + 50	
			50 + 100	
			100 + 200	
			200 + 400	
			400 + 800	
Florasulam + clopyralid	Primus Perfect	WG	0.47 + 5.6	Corteva Agriscience Hellas, Athens, Greece
			0.94 + 11.2	
			1.88 + 22.4	
			3.76 + 44.8	
			7.52 + 89.6	
			15.04 + 179.2	
			30.08 + 358.4	

[a]Abbreviations: SG, water-soluble granules; WG, water-dispersible granule; SL, soluble liquid.
[b]Tribenuron and tribenuron + mecoprop-P were applied with the surfactant Trend® 90 SL (Corteva Agriscience, Athens, Greece) at 0.1% v/v; pyroxsulam + florasulam was applied with the surfactant Biopower® SL (Bayer CropScience, Athens, Greece) at 0.33% v/v; imazamox was applied with the surfactant Dash® HC (BASF Hellas, Athens, Greece) at 0.4% v/v.
[c]The rates in boldface are the label recommended rates of the herbicides.

of the isolated DNA were checked using a NanoPhotometer™ Pearl (Implen, Munich, Germany). The amplification of the *ALS* gene fragment from the genomic DNA samples, containing the Pro-197, Asp-376, and Trp-574 codons (1,602 bp), was achieved using the forward 5′-AGGTGGAGCTTCAATGGAGA-3′ and reverse 5′-CCTGCAGGAATCATGGGTAA-3′ primer pair (Hada et al. 2021). A 50-µl PCR reaction volume was set up containing 1× Kapa Taq Buffer A, 200 µM of each dNTP (Jena Bioscience, Germany), 600 nM of each primer, 0.25 units of Kapa Taq DNA Polymerase (Kapa Biosystems, Wilmington, MA), 40 ng of template DNA, and nuclease-free water. Thermal cycling conditions consisted of an initial denaturation step of 95 C for 3 min followed by 35 cycles of 95 C for 30 s, 60 C for 30 s, and 72 C for 1 min 45 s, with a final extension at 72 C for 5 min. The PCR amplicons were analyzed in 2% agarose gels and purified with QIAEX II Gel extraction Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The purified PCR products were single-strand sequenced with BigDye Terminator v3.1 (Life Technologies, Waltham, MA) cycle sequencing methodology, on an ABI3730 Genetic Analyzer (Applied Biosystems™, Waltham, MA), using the same primers as for PCR and an additional internal forward primer 5′-ATGGGTCTTGGGACTTTTCC-3′. To detect the presence or absence of point mutations of the *ALS* gene, corn marigold sequences of R and S plants were manually checked, aligned, and

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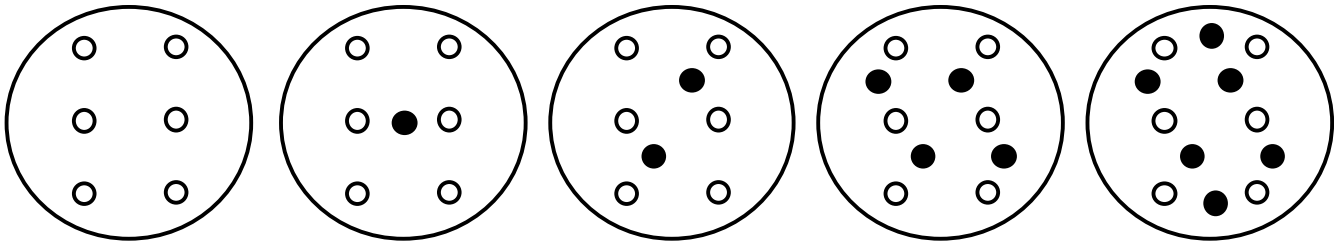

Figure 1. Schematic presentation of the density pattern (6:0, 6:1, 6:2, 6:4, 6:6) to assess plant responses of the R2, R3, and S corn marigold populations grown in competition with barley Open circles, barley; black circles, R or S corn marigold populations.

compared to *Arabidopsis thaliana* nucleotide sequence for *ALS* gene (GenBank Accession Number: X51514), using BioEdit v7.2.5 software (Hall 1999).

Competitive Ability of R2, R3, And S Corn Marigold Populations Against Barley

A target-neighborhood design was used to evaluate the plant response of the R2, R3, and S corn marigold populations grown in competition with barley (cv. 'Thessaloniki'). The R1 population was not included in this study, as its response to ALS-inhibiting herbicides was similar to that of the R2 population. More specifically, the experiment was conducted in plastic pots (20 × 25 × 30 cm) at the University of Western Macedonia farm in Florina, Greece, during late January to late May of the 2021 growing season. The pots were filled with soil having the characteristics described above in the whole-plant dose–response pot experiment. Each pot was seeded with barley in two rows spaced 15 cm apart, having three hills (two seeds per hill) spaced 8 cm per row. Then, 0 (weed-free crop control), 1, 2, 4, and 6 corn marigold seedlings, at the two-leaf stage, were transplanted into each pot (Figure 1), when barley plants were also at the two-leaf stage (after careful thinning to leave one barley plant per hill). The weed density studied corresponds to 20, 40, 80, and 120 plants m^{-2}. All pots were placed outdoors in a net-protected area for 90 d (late February to late May), where they were irrigated and fertilized to maintain vigorous growth throughout the experiment. Emerging grass and broadleaf weeds were carefully removed manually during the duration of the experiment to assure absence of competition arising from the presence of other weeds.

Plant growth was evaluated by determining the aboveground biomass (fresh weight) of the six barley plants, along with the aboveground biomass of corn marigold plants per pot. Each experiment was conducted twice, using a randomized complete block design with three replications per treatment.

Statistical Analysis

Fresh-weight data of the whole-plant dose–response assays to ALS inhibitors and of the whole-plant dose–response assays to ALS inhibitors or ALS mixtures with synthetic auxin herbicides were expressed as a percent reduction from the untreated control and subjected to ANOVA. ANOVA combined over two runs was performed for the three suspected R or S populations to test for treatment by experimental-run interaction. The data were analyzed over the two experiments, because the homogeneity of variances checked by using Bartlett's test (Snedecor and Cochran 1989) indicated no significant departure from normality. Means were separated with Fisher's protected LSD test at $\alpha = 0.05$. In addition, the combined growth response data were also fit to a four-parameter log-logistic curve for nonlinear regression analysis (Seefeldt et al. 1995):

$$y = c + (d - c)/\{1 + \exp[b(\log x - \log GR_{50})]\} \quad [1]$$

where c is the lower limit, d is the upper limit, and b is the relative slope around the herbicide dose resulting in 50% growth reduction (GR_{50}). The herbicide dose was the independent variable (x), and the growth response (fresh-weight reduction % of the untreated control) was the dependent variable (y) in the regression equation. This equation was chosen, as similar studies have used it to accurately estimate the dose causing a 50% fresh-weight growth reduction (GR_{50}).

The data obtained from the competition study between the R2, R3, and S corn marigold populations and barley were analyzed over the two runs, because the homogeneity of variances checked by Bartlett's test (Snedecor and Cochran 1989) indicated no significant departure from normality. The 3 (populations) × 5 (crop/weed densities) factorial approach was used for barley, whereas the 3 (populations) × 4 (weed densities) factorial approach was used for corn marigold. Moreover, the pooled-over two experiments aboveground biomass data of either barley or corn marigold were used for linear regression against weed density, where the aboveground biomass of barley or corn marigold plants were the dependent variables (y) and the weed density was the independent variable (x). The estimated b slopes were compared with a t-test at $P = 0.05$. All statistical analyses were performed using SPSS v.23 (IBM, Chicago, IL).

Results and Discussion

Whole-Plant Dose–Response Assays to ALS Inhibitors

The three putative R corn marigold populations were not effectively controlled by most of the tribenuron, pyroxsulam + florasulam, and imazamox rates used (Figure 2). In particular, the tribenuron tested rates reduced fresh weight of the R1, R2, and R3 populations by 8% to 50%, 4% to 40%, and 0 to 35%, respectively, whereas the respective reduction due to pyroxsulam + florasulam was 44% to 99%, 36% to 82%, and 23% to 58%. Also, the application of imazamox rates reduced fresh weight of R1, R2, and R3 populations by 35% to 100%, 52% to 100%, and 24% to 59%, respectively. In general, fresh weight of all R populations was reduced less by tribenuron as compared to pyroxsulam + florasulam and imazamox, whereas fresh weight of R3 population was reduced less by all herbicides tested as compared to R1 and R2 populations. By contrast, all rates tested of the above ALS-inhibiting herbicides reduced fresh weight of the S population by 100%.

The calculated GR_{50} values (herbicide rate required for 50% fresh-weight reduction) for the R1 and R2 populations to

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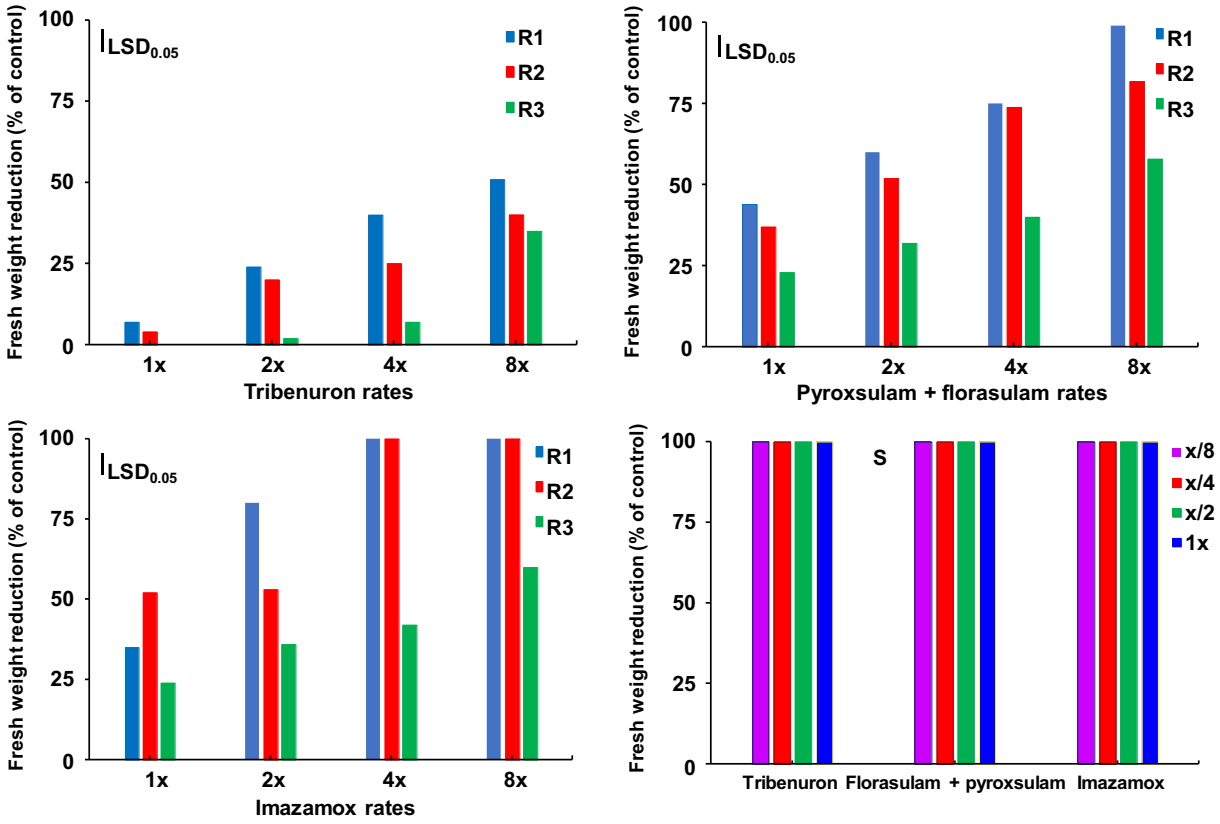

Figure 2. Fresh-weight reduction (% of untreated control) of the R1, R2, R3, and S corn marigold populations due to application of the ALS-inhibiting herbicides tribenuron, pyroxsulam + florasulam, and imazamox. The recommended (1×), 2×, 4×, and 8× rates were used for the R populations, and the ×/8, ×/4, ×/2, and 1× for the S population. Values are the means of six replicates over two runs. LSD allows comparison across treatments. F = 36.18 and P = 0.001 for R populations.

tribenuron were 106 and 179 g ha^{-1}, respectively, whereas the GR_{50} value for the R3 was not calculated because the highest rate of tribenuron reduced fresh weight by 35% (Table 2). The pyroxsulam + florasulam GR_{50} values for the R1, R2, and R3 populations were 28, 26, and 115 g ha^{-1}, respectively, whereas the respective imazamox GR_{50} values were 67, 85, and 289 g ha^{-1}. The GR_{50} values for the S population could not be calculated, because all herbicide rates reduced its fresh weight by 100%, and thus a resistance index could not be determined. Generally, the R3 population was affected less by all herbicide treatments compared with the R1 and R2 populations.

The unsatisfactory control of the R1, R2, and R3 corn marigold populations with tribenuron, pyroxsulam + florasulam, and imazamox applied at rates higher than the recommended field rates strongly supports the evolution of cross-resistance to ALS inhibitors in these populations. These findings agree with those reported by Hada et al. (2020), who found cross-resistance to the ALS-inhibiting herbicides florasulam, imazamox, and tribenuron in a closely related weed species, crown daisy (*Glebionis coronaria* L.). Tal and Rubin (2004) also reported cross-resistance in field-selected crown daisy populations to ALS-inhibiting herbicides.

Whole-Plant Dose–Response Assays to ALS Mixtures with Synthetic Auxin Herbicides

The tribenuron + mecoprop-P tested rates reduced fresh weight of the R1, R2, and R3 populations by 74% to 100%, 51% to 100%, and 60% to 100%, respectively (Figure 3), whereas the respective reduction due to florasulam + aminopyralid was 97% to 100%, 92% to 100%, and 93% to 100% (Figure 3). Moreover, the fresh weight of the R1, R2, and R3 populations was reduced due to tritosulfuron + dicamba by 37% to 100%, 22% to 100%, and 46% to 100%, whereas the respective reduction due to florasulam + clopyralid was 64% to 100%, 31% to 100%, and 50% to 100%. Averaged across R populations and herbicide treatments, the efficacy of mixtures in increasing order was tritosulfuron + dicamba < florasulam + clopyralid < tribenuron + mecoprop-P < florasulam + aminopyralid, whereas their respective efficacy against the S population was excellent, as both the half and recommended label rate of all herbicide mixtures provided 100% control of this weed. The susceptibility of the R populations in increasing order, averaged across herbicide treatments, was R2 < R1 = R3. In contrast to these results, Hada et al. (2020) found that the co-mixtures of aminopyralid + florasulam + 2,4-D and aminopyralid + florasulam provided lower efficacy (85%) against crown daisy than that obtained in our study and by their application of co-mixtures synthetic auxin herbicides 2,4-D + MCPA and dicamba + 2,4-D (respective efficacy of 92% and 94%). In addition, Hada et al. (2022) found in another field study that the application of 2,4-D + florasulam on cereals and clopyralid on rapeseed reduced crown daisy densities by 44%, 75%, and 66% in wheat, barley, and rapeseed, respectively, whereas the respective reduction due to dicamba + 2,4-D was 85% and 91% in wheat and barley growth. These efficacy differences could be attributed to differing activity between the synthetic auxin herbicides, differing compatibility in co-formulations with ALS inhibitors, differing susceptibility among the R populations to these herbicides, differing competitive or allopathic ability of the crop

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Table 2. Dose–response model parameter estimates, GR_{50} values (g ai ha^{-1}), *b* slopes, and R^2 for the three R corn marigold population in response to tribenuron, pyroxsulam + florasulam, and imazamox. Models were generated utilizing % fresh-weight reduction from the respective untreated controls. Dose–response curves were not generated for the S population, because at the lowest test rate for each herbicide, fresh weight was reduced by 100%.

Tribenuron			
Populations	GR_{50} (95% CI)[a]	*b* Slope (SE)	R^2
R1	106 (87–112)	2.29 (0.17)	0.93
R2	179 (152–234)	2.2 (0.22)	0.91
R3	NA[b]		
Pyroxsulam + florasulam			
R1	28 (27–28)	6.93(0.38)	0.99
R2	26 (23–36)	3.56(0.63)	0.76
R3	115 (98–128)	1.62(0.1)	0.88
Imazamox			
R1	67 (62–71)	2.91 (0.24)	0.93
R2	85 (82–93)	2.43 (0.13)	0.97
R3	289 (265–310)	1.67 (0.08)	0.95

[a]Abbreviation: GR_{50}, Tribenuron, pyroxsulam + florasulam, and imazamox concentration (g ai ha^{-1}) for 50% reduction of the corn marigold fresh weight.
[b]NA, GR_{50} value was not estimated for tribenuron, as the highest rate reduced fresh weight <50%.

species against the weed populations, and differing environmental conditions prevailing during the experiments. Based on these findings, further research is needed to see if these results are applicable to other ALS-resistant corn marigold populations.

ALS Gene Sequencing

Nucleotide and amino acid sequence alignment of the amplified *ALS* gene fragment from the S, R1, R2, and R3 corn marigold plants showed that all sequenced R plants contained a single homozygous point mutation or two coexisting heterozygous point mutations (Figure 4). More specifically, all four sequenced plants from the R1 population were homozygous for a cytosine-to-adenine substitution at the codon Pro-197, resulting in amino acid change to Thr. The same point mutation was also observed in three out of four R2 homozygous plants, whereas the fourth plant contained two coexisting heterozygous mutations at positions Pro-197-Thr and Trp-574-Leu. Finally, two of the four R3 homozygous plants had the Pro-197-Thr substitution, whereas the other two R3 homozygous plants contained the Trp-574-Leu substitution.

The detected Pro-197-Thr or Trp-574-Leu substitutions or the two coexisting mutations in the *ALS* gene are responsible for the cross-resistance of the R1, R2, and R3 corn marigold populations to chemically dissimilar ALS inhibitors and confirm the results found in the whole-plant dose–response assays to herbicides. Similar results were reported by Hada et al. (2021), who found that the amino acid substitution of Pro-197 to Thr, Ser, Gln, Arg, or the Asp-376 to Glu or the Trp-574 to Leu substitution in two crown daisy populations were responsible for cross-resistance to ALS inhibitors bispyribac, florasulam, imazamox, and tribenuron. It is worth noting that the cross-resistance of corn marigold and crown daisy populations to imazamox due to Pro-197 substitution was not expected, as sulfonylurea herbicides (e.g., tribenuron) predominantly select for Pro-197 mutation, whereas sulfonylurea and imidazolinone (e.g., imazamox) herbicides predominantly select for Trp-574-Leu (Heap 2022; Ntoanidou et al. 2019; Zhao et al. 2020).

The detected Trp-574-Leu substitution in the *ALS* gene in two of the four sequenced R3 plants (in addition to the Pro-197-Thr substitution in the other two plants) could explain the less affected growth of this population by all rates of tribenuron, pyroxsulam + florasulam, and imazamox as compared to the R1 and R2 populations. Beckie and Tardif (2012) also reported that the Trp-574-Leu substitution confers high level and broad-spectrum cross-resistance to all chemically dissimilar classes of ALS-inhibiting herbicides across several weed species. In addition, various cross-resistance patterns to ALS-inhibiting herbicides due to target-site mutations in the *ALS* gene were reported by Yu and Powles (2014) and Varanasi et al. (2018). The coexisting Pro-197-Thr and Trp-574-Leu mutations in the *ALS* gene of corn marigold plants have also recently been discovered by Deng et al. (2017) in another weed, flixweed (*Descurainia sophia* L.).

The agreement between (i) the GR_{50} values calculated from the whole-plant rate–response experiments and (ii) the detected point mutation at codons Pro-197 or/and Trp-574, supports the evidence that the reduced activity of tribenuron, florasulam + pyroxsulam, and imazamox against all R populations could be attributed to alteration of the ALS binding site, which eventually reduces its affinity to ALS-inhibiting herbicides. Regarding the differences between the GR_{50} values of the R populations, these could be chiefly attributed to varying frequencies of the individuals with homozygous and heterozygous different point mutations.

Competitive Ability of S, R2, or R3 Corn Marigold Populations Against Barley

The target–neighbor design was used to evaluate the competitive effect of the S and R populations on barley, because this design provides both the target competitive effect on its neighbors and the target's reaction to the neighbors (Barry and Dudash 2015). As the ANOVA indicated that aboveground biomass of barley was significantly affected by weed populations, weed density, and their interaction, the population-by-density results are presented. More specifically, barley plants grown in competition with one, two, four, and six plants of the R2 population displayed 8%, 24%, 32%, and 45% reduction in aboveground biomass, respectively, as compared with the weed-free barley, whereas the respective reduction due to competition of the R3 population was 8%, 32%, 37%, and 44% (Figure 5). In addition, aboveground biomass of barley plants was reduced by 13%, 20%, 35%, and 46% due to the presence of one, two, four, and six plants of the S population. The high R^2 values (0.96, 0.98, and 0.87) for the linear regression performed between barley aboveground biomass against weed density of the S, R2, and R3 populations, respectively, suggests proportional reduction of barley with increasing weed density. The calculated similar negative slopes (–18.9, –18, and –16.1) for barley grown in competition with S, R2, and R3 populations, using *t*-test at $P = 0.05$, show clearly that competitive ability of barley was similar when grown with either the S or R weed populations. In contrast to these results, Hada et al. (2020) reported that wheat yield regressed against crown daisy density ranging from 20 to more than 100 plants m^{-2} followed a sigmoidal curve, with wheat yield reduction reaching 75% for the higher weed density. These differences in competitive ability could be attributed to different weed and crop species, different weed populations, and different environmental conditions prevailing during the experiments.

Aboveground biomass of the S, R2, and R3 populations grown in competition with barley was proportionally increased with increasing density (Figure 5). This is confirmed by the high R^2 values (0.98, 0.99, and 0.99) of the linear regression performed between aboveground biomass of the S, R2, and R3 populations

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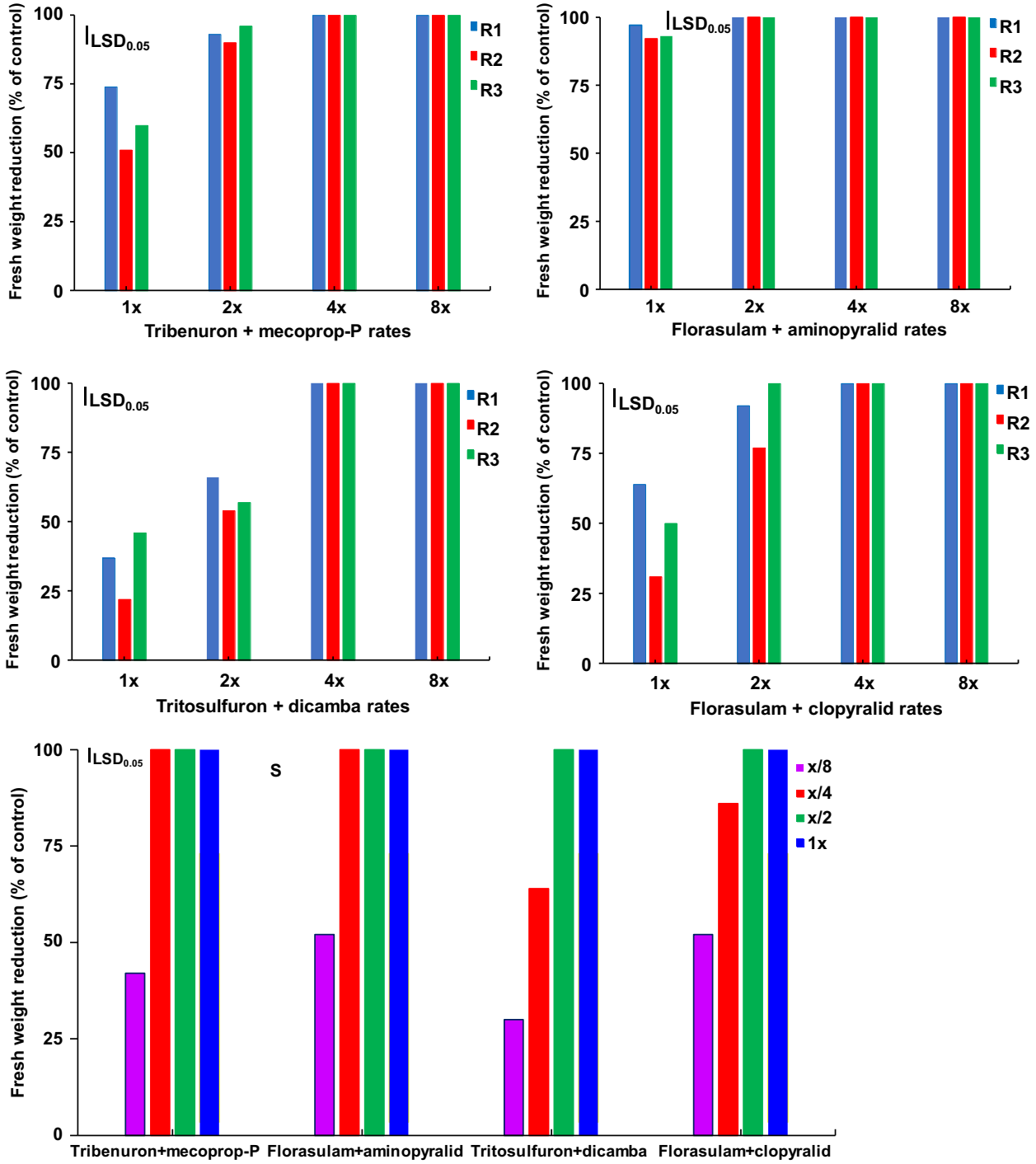

Figure 3. Fresh-weight reduction (% of untreated control) of the R1, R2, R3, and S corn marigold populations due to application of the ALS plus synthetic auxin herbicides tribenuron + mecoprop-P, florasulam + aminopyralid, tritosulfuron + dicamba, and florasulam + clopyralid. The recommended (1×), 2×, 4×, and 8× rates were used for the R populations, and the ×/8, ×/4, ×/2, and 1× for the S population. Values are the means of six replicates over two runs. LSD allows comparison across treatments. F = 75.38 and P = 0.001 for R populations; F = 379.17 and P = 0.001 for S population.

against weed density. The estimated similar positive slopes (37.8, 35.2, and 32.7) for the S, R2, and R3 populations grown in competition with barley, according to *t*-test at P = 0.05, supports the lack of a negative association between the competitive ability of these R populations and the resistance of the ALS target-site enzyme. In contrast to these results, Vercellino et al. (2021) found that the R plants (due to a Trp-574 mutation in the *ALS* gene) of the feral radish (*Raphanus sativus* L.), grown under wheat competition, produced 36% to 46% less total aboveground biomass, 26% to 47% fewer seeds per plant, and 36% to 53% less plant yield compared to S plants. Palmieri et al. (2021) detected a decrease in catalytic activity and reduced substrate affinity due to Trp-574-Leu substitution in the *ALS* gene of the ALS-resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.) populations as compared with the S population. However, Kaloumenos et al. (2012) found that the growth of four R rigid ryegrass (*Lolium*

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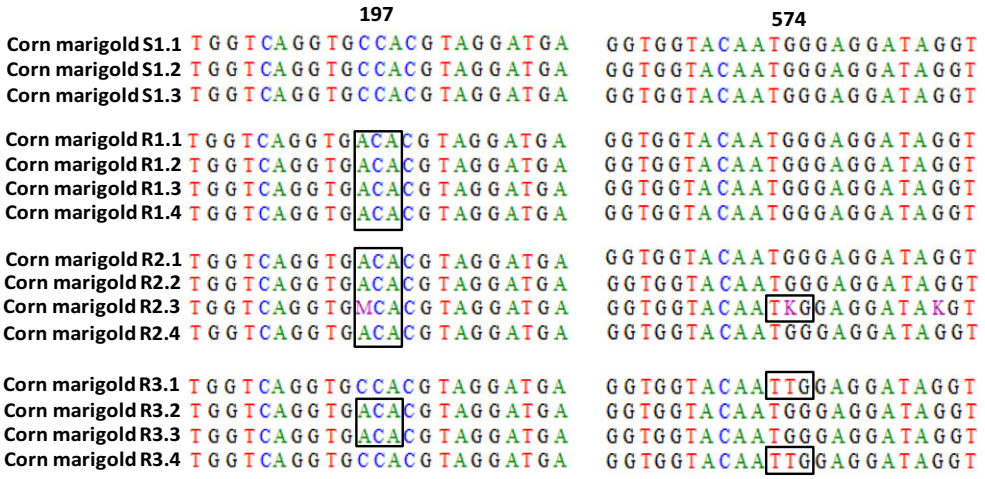

Figure 4. Nucleotide sequence alignment of the *ALS* gene, taken from plants of the S, R1, R2, and R3 corn marigold populations. S, susceptible; R, resistant. The codons refer to the standard *Arabidopsis thaliana ALS* gene (GenBank: X 51514). The observed point mutations at codon 197 and 574 are in boxes. IUPAC-IUB nucleotide codes: CCA, proline; ACA, threonine; TGG, tryptophan; TTG, leucine; MCA, CCA/ACA (Pro/Thr); TKG, TGG/TTG (Trp/Leu).

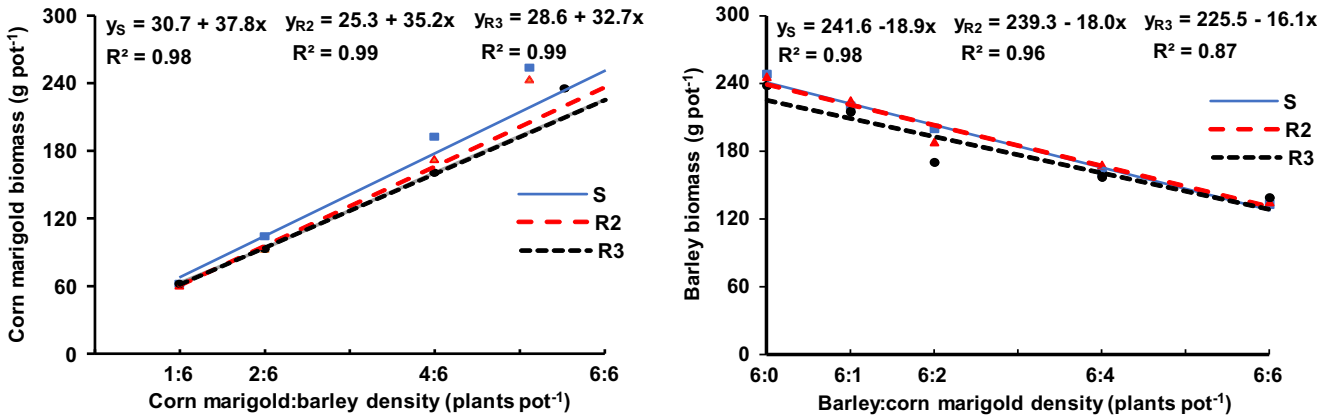

Figure 5. Linear equations and coefficient of determination for the aboveground biomass (fresh weight) of the S, R2, and R3 corn marigold populations or barley grown in competition and regressed against weed density.

rigidum L.) populations to ALS inhibitors (due to Pro-197 substitution by Ala, Arg, Gln, Leu, or Ser and probably His or Val) in the absence of crop competition was similar to that of four S populations, suggesting that the resistance endowing *ALS* mutations did not result in detectable resistance adaptation cost. The above unchanged or reduced ALS enzyme activity or growth parameters determined in different weed species resistant to ALS inhibitors due to specific amino acid substitutions, suggests the necessity of case-by-case analysis to investigate and determine the precise impact of specific gene polymorphisms on ALS kinetics and possible pleiotropic effects (Yu et al. 2010; Zhao et al. 2020). Moreover, the similar competitive ability between S and R weed populations could be attributed to (i) the quantitative inheritance of most of the weed growth traits and (ii) the strong influence of environmental variance and genotype-by-environment interactions, which usually obscure and hinder the full and clear expression of their genotype differences (Coleman et al. 2001; Worthington and Reberg-Horton 2013).

The reduced control of the R1, R2, and R3 corn marigold populations after the application of the ALS-inhibiting herbicides tribenuron, pyroxsulam + florasulam, and imazamox, along with the *ALS* gene sequence, support the evidence of evolved target-site cross-resistance due to Pro-197-Thr or/and Trp-574-Leu amino acid substitutions in the *ALS* gene. That the recommended rate of the co-formulated herbicide mixture florasulam + aminopyralid was very effective against the R populations of this weed suggests its possible use as an alternative chemical option for their control, but this was not the case for the recommended rates of tritosulfuron + dicamba, florasulam + clopyralid, and tribenuron + mecoprop-P. The similar aboveground biomass of the S and R populations grown in competition with barley suggests a lack of association between the competitive ability of the R corn marigold populations and target-site resistance mechanism. Based on these results, a long-term integrated weed management strategy should be adopted, utilizing diversified control tactics such as crop rotation, lack of selection pressure from ALS inhibitors, and application of alternative herbicides with different modes of action or complementary methods to mitigate field-selection, establishment, and spread of ALS R corn marigold populations.

Practical Implications

Corn marigold is an annual cross-pollinated branched dicot species that preferentially infests winter cereals, where it forms

https://doi.org/10.1017/wet.2023.17 Published online by Cambridge University Press

dense stands resulting in significant reduction in crop productivity. Control of this weed in winter cereals has mainly relied on ALS inhibitors, which are the most prone to rapid evolution of target-site mediated resistance. However, as some corn marigold populations in winter cereals grown in central Greece have evolved cross-resistance to the ALS-inhibiting herbicides tribenuron, pyroxsulam + florasulam, and imazamox, serious measures for the management of this weed are needed. That the recommended rate of the co-formulated herbicide mixture florasulam + amino-pyralid was very effective against the R corn marigold populations suggests its possible use as an alternative chemical option for their control, but this was not the case for the recommended rates of tritosulfuron + dicamba, florasulam + clopyralid, and tribenuron + mecoprop-P. These efficacy differences between the synthetic auxin herbicides could be attributed to differing activity, varying compatibility in co-formulations with ALS inhibitors, and differing susceptibility to these herbicides among the resistant populations. Based on these results, a long-term integrated weed management strategy should be adopted, utilizing diversified control tactics such as crop rotation, lack of selection pressure from ALS inhibitors, and application of alternative herbicides with different modes of action or complementary methods to mitigate field-selection, establishment, and spread of ALS-resistant corn marigold populations.

Acknowledgments. We thank Dr Eleni Anthimidou and Dr Symela Ntoanidou who participated in data analysis, use of software and visualization of results. We also thank the Associate Editor and two anonymous reviewers for their constructive comments, which helped us to improve the manuscript. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. No conflicts of interest have been declared.

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