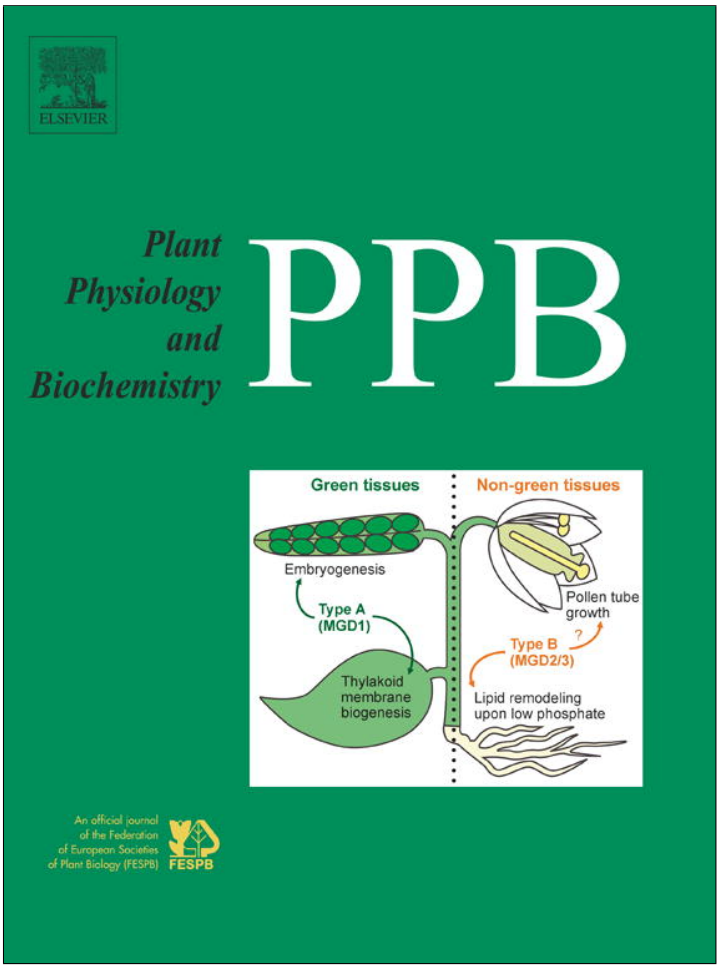

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Short communication

Acetohydroxyacid synthase (AHAS) *in vivo* assay for screening imidazolinone-resistance in sunflower (*Helianthus annuus* L.)

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ABSTRACT

The objective of this work was to evaluate the *in vivo* acetohydroxyacid synthase (AHAS) activity response to imidazolinones and its possible use as a selection method for evaluating AHAS inhibitor resistance. *In vivo* AHAS assay and the comparison of parameters from dose–response curves have been used as a valid tool for comparing sunflower lines and hybrids differing in imidazolinone resistance. The sunflower resistant genotypes evaluated here were 100-fold and 20-fold more resistant compared with the susceptible line for imazethapyr and imazapyr, respectively. This assay also allowed discrimination of homozygous from heterozygous genotypes for I_{mr1} *locus* that codify for the catalytic subunit of AHAS. The *in vivo* AHAS assay described in this study was useful for the selection of sunflower genotypes differing in herbicide resistance and could be a useful tool when breeding for imidazolinone resistance in sunflower.

1. Introduction

Acetohydroxyacid synthase (AHAS; EC 4.1.3.18) is the first enzyme in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine. This enzyme catalyzes two reactions: for valine and, leucine biosynthesis two pyruvate molecules are condensed to form 2-acetolactate, while for isoleucine, 2-acetohydroxybutyrate is synthesized from pyruvate and 2-ketobutyrate. AHAS is also known as acetolactate synthase (ALS) but this name is not preferred because it ignores the role of the enzyme in acetohydroxybutyrate synthesis [1,2].

AHAS is the target site of several herbicides including five structurally diverse chemical classes: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthio (or oxy)-benzoates (POB) and sulfonylamino-carbonyltriazolinones (TZ) [3,4]. These herbicides inhibit AHAS by blocking a channel through which substrates access the active site [5,6].

Despite the increased knowledge of AHAS inhibition, the mode of action of these herbicides is not fully understood. Mechanisms implicated in plant death include amino acid starvation, toxic compound accumulation, disruption of protein synthesis and disruption of photosynthate transport [7–9]. In certain crops such as *Glycine max* and *Triticum aestivum*, the selectivity of some AHAS inhibitor herbicides is based primarily on the ability of the crop to metabolize the herbicide [3,10,11]. But, in general, herbicide resistance is originated from an alteration in the target site of the enzyme [12–14].

Since the commercial launch of imidazolinone (IMI) tolerant maize in 1992, five other IMI resistant crops (oilseed rape, lentil, rice, wheat and sunflower) have been developed and commercialized using conventional breeding methods [15,16]. In sunflower, IMI resistance is due to a form of the AHAS large subunit (AHASL) that is less sensitive to herbicide inhibition.

Bruniard and Miller [17] proposed a digenic model in which a major semidominant gene (I_{mr1}) interacting with a second modifier gene (I_{mr2}) confers resistance to IMIs in sunflower. Consequently, complete resistance in sunflower can only be achieved by homozygosity of both resistant genes ($I_{mr1}I_{mr1}I_{mr2}I_{mr2}$) in an inbred line or hybrid [17]. Based on molecular studies, Kolkman et al. [18] identified and characterized three genes coding for the AHAS catalytic subunits in sunflower (*Ahasl1*, *Ahasl2 and Ahasl3*). *Ahasl*1 is a multiallelic locus and the only member of this small family where all the induced and natural mutations for herbicide resistance were described in sunflower. The first mutation at *Ahasl1* conferring resistance to IMI was discovered in a wild population (PUR *Helianthus annuus*) in Kansas, USA, and was identificated *Ahasl1-1* (also known as I_{mr1} or Ar_{pur}) and has been introgressed to elite inbred lines [19]. The effect of the modifier *locus*, I_{mr2} remains

Abbreviations: AHAS, acetohydroxyacid synthase; CPCA, 1,1-cyclopropanedicarboxylic acid; I_{50}, herbicide dose that inhibited AHAS by 50%; IMI, imidazolinone; KARI, keto-acid reductoisomerase.

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unclear and it might be related to non-target-site resistance [18,20]. The development of IMI resistant cultivated sunflower represents a major step in advancing weed control for this species [21].

More recently, a second resistance gene was developed by mutagenesis and selection with imazapyr [22]. This nuclear, partially dominant allele was named *Ahasl1-3* and confers superior IMI resistance than *Ahasl1-1* [23,24]. Sala and Bulos [25] described a third resistance gene, *Ahasl1-4*, from a natural-occurring population in Jovita (Córdoba, Argentina). *Ahasl1-4* presents a pattern of cross-resistance to different AHAS-inhibiting herbicides (IMI, SU, TZ and POB). To date *Ahasl1-1* and *Ahasl1-3* alleles are being routinely introgressed for the production of sunflower hybrids tolerant to imidazolinones.

The development of resistant elite inbred lines requires the introgression of resistance genes into elite germplasm by backcrossing. The implementation of this process requires identification of IMI resistant genotypes which involves herbicide application to plants grown in the field or greenhouse, being a time-consuming and costly task. The development of a laboratory technique that allows early screening could be a useful tool to help reducing time and resources when breeding for IMI resistance.

Among the different types of diagnostic tests, soil-less assay is one of the most attractive because of the large amount of individuals screened in a limited space [26]. In particular, *in vivo* AHAS assay is an efficient laboratory method for quickly identifying herbicide resistance [27,28]. As opposed to the *in vitro* techniques performed on the isolated enzyme in a test tube, the *in vivo* AHAS assay uses an inhibitor of the ketoacid reductoisomerase, EC 1.1.1.86 (KARI), the enzyme catalyzing the next step in the branched-chain amino acid biosynthetic pathway. Inhibition of KARI results in an accumulation of the products of the AHAS catalyzed reactions. In the presence of an AHAS inhibitor, carbon flow from pyruvate is inhibited in susceptible plants and no acetohydroxyacid is produced. AHAS activity is indirectly measured by converting AHAS products to chromophores that are then quantified colorimetrically [29].

The aim of this work was to evaluate the *in vivo* AHAS activity response to imidazolinones and its possible use as a selection tool for evaluating AHAS inhibitor resistance among sunflower lines and their hybrids.

2. Results and discussion

In vivo AHAS assay is an efficient method for monitoring and assessing AHAS inhibitors resistance and is based on the accumulation of products in the presence of an AHAS inhibitor herbicide. Estimated dose–response curves for sunflower *in vivo* AHAS activity in response to imazapyr and imazethapyr are presented in Fig. 1. The lack-of-fit *F*-tests were not statistically significant ($p > 0.05$), meaning that the log–logistic model with three parameters provided an acceptable description of the data.

The sunflower resistant genotypes ($I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ and $I_{mr1}I_{mr1}I_{mr2}i_{mr2}$) had I_{50} values of approximately 100 μM and were 100-fold and 20-fold more resistant compared with the susceptible line ($i_{mr1}i_{mr1}i_{mr2}i_{mr2}$) for imazethapyr and imazapyr, respectively (Table 2). These values are in the same order of magnitude than those previously reported on the same species. White et al. assessed *in vivo* AHAS activity on an imazethapyr-resistant sunflower biotype founded in Howard, South Dakota, that showed an I_{50} of 200 μM (39-fold more resistant than the susceptible biotype) [30]. Other researchers found that *in vivo* AHAS

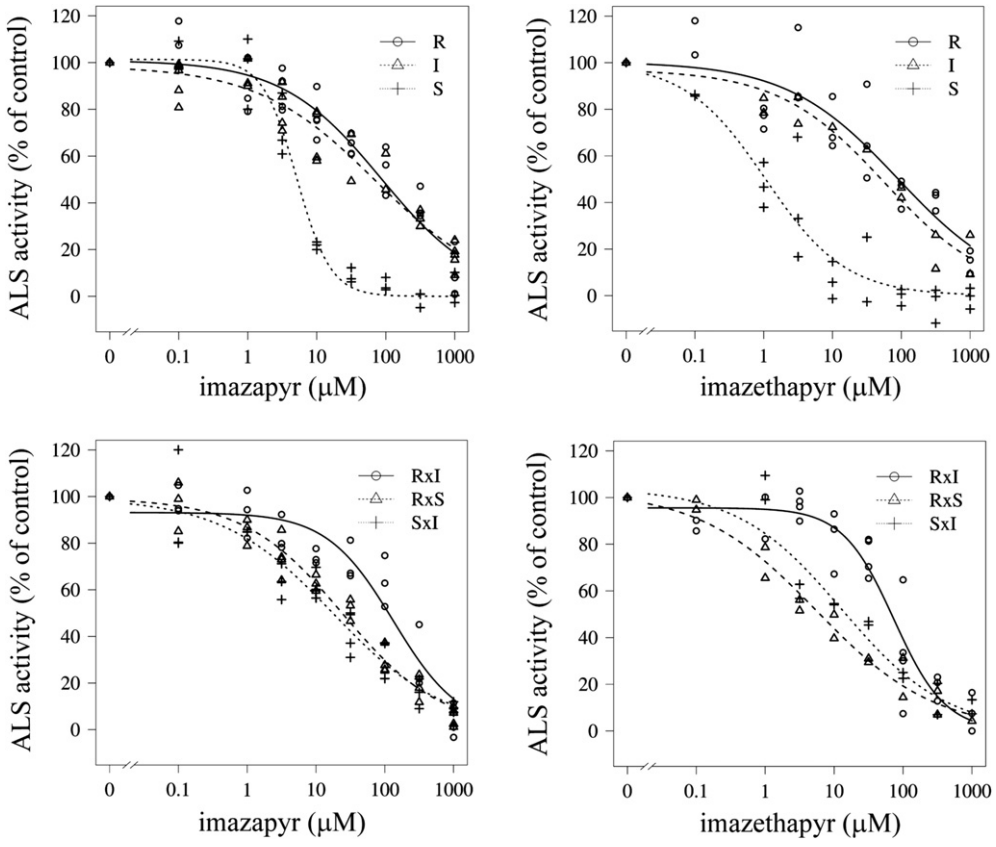

Fig. 1. Dose–response curves of *in vivo* acetohydroxyacid synthase (AHAS) activity expressed as percentage of the control for resistant (R), intermediate (I), susceptible (S) inbred lines and their F_1 hybrids.

inhibition by imazethapyr was 210 folds higher in resistant sunflower hybrid when compared to susceptible hybrid [31].

Previous research on imidazolinone resistant wild sunflower has shown similar results at the enzyme level. *In vitro* AHAS assays found that herbicide concentrations required to inhibit enzyme activity by 25% were 332 and 210 times greater in the resistant biotype than in the sensitive biotype for imazamox and imazethapyr, respectively [20,32]. Resistance levels obtained from *in vitro* activity are in general higher than those obtained from *in vivo* assays [33]. Differences between the *in vivo* and *in vitro* assays are due to a combination of physical barriers between the site of application and the intracellular target, degradation and detoxification by the plant [30]. For this reason, *in vitro* AHAS activity would be an inaccurate predictor of whole plant responses to AHAS inhibitor herbicides.

Based on the I_{50} values, the resistant line ($I_{mr1}I_{mr1}I_{mr2}I_{mr2}$) was not different from the intermediate line ($I_{mr1}I_{mr1}i_{mr2}i_{mr2}$) and the hybrid between them ($I_{mr1}I_{mr1}I_{mr2}i_{mr2}$). By the other hand, they showed differences in the slope parameter value (b) (Table 3). It is interesting that these genotypes are homozygous for I_{mr1} but differ on the constitution of I_{mr2} *locus*. Therefore, differences in b values can be ascribed to I_{mr2} while I_{50} in this scenario could be associated with I_{mr1}.

Correspondingly, I_{50} values did not differ between genotypes that were heterozygous for I_{mr1} but showed statistically significant differences among genotypes that differed at the I_{mr1} *locus* (Table 3). I_{mr1} is an allelic variant of the *Ahasl1-1 locus* that codes for the AHAS catalytic subunit and harbors a C-to-T mutation on codon 205 [18]. Hence, I_{50} values explain herbicide-sensitivity differences that could be associated to the phenotypic expression of I_{mr1} *locus*.

The results presented here suggest a connection between I_{mr2} and the b parameter. Although there were differences between the intermediate line ($I_{mr1}I_{mr1}i_{mr2}i_{mr2}$) and the resistant hybrid ($I_{mr1}I_{mr1}I_{mr2}i_{mr2}$), the intermediate and resistant lines did not differ (Table 3). At whole plant level, our previous research showed that these genotypes could be discriminated [34]. The type of gene action of I_{mr2} could not be evaluated using the *in vivo* AHAS assay presented in this work. The effect of the modifier *locus*, I_{mr2}, remains unknown but it could be related to non-target site resistance such as herbicide uptake, translocation or metabolism. Modifications of the *in vivo* AHAS assay that allow the quantification of herbicide metabolism and absorption on enzyme activity could contribute to a better understanding of IMI resistance in cultivated sunflower. The *in vivo* AHAS assay proposed by Simpson et al. would give more information in this manner [28,35]. It was found that the AHAS *in vivo* activity from foliar application of KARI inhibitor are higher than those reported by Gerwick et al. for isolated tissues incubated in a KARI inhibitor solution. It can be hypothesized that foliar applications result in less disruptions of the intact biological system compared to incubation of detached leaves [27,28].

There were differences between the two imidazolinone herbicides although they cannot be compared statistically as they were tested in separate experiments (Fig. 1, Table 2). As reported previously, imazethapyr is a stronger inhibitor than imazapyr [36]. The two herbicides have a pyridine ring (besides the imidazole moiety) in their molecular structure but differ at the position five of the pyridine ring. This difference is thought to play a small role in inhibition of AHAS. Instead, the functional groups are related to certain characteristic such as metabolism in plants [15,16,37]. Nevertheless, additional experiments would be necessary to confirm this hypothesis. Imidazolinone-tolerant plants metabolize imazapyr to a relative immobile metabolite which prevents translocation of the herbicide to the growing points of plants. Unlike imazapyr, imazethapyr is metabolized to nontoxic forms via hydroxylation of the ethyl substituent followed by conjugation to glucose [37]. Imazapyr metabolism to a non-toxic low mobility compound was previously noticed in sunflower [38]. It is important to consider that this *in vivo* AHAS assay could detect differences between these two herbicides.

We used a rapid *in vivo* AHAS assay to study IMI resistance in sunflower but it is also applicable to a number of weed and crop species. It could be used to rapidly identify resistant biotypes grown in field or in greenhouse. An additional positive attribute is the presence of an internal control in the diagnosis, that ensures the validity of the method [27]. *In vivo* AHAS assay and dose–response curves-parameter comparison contributed to the characterization of IMI resistance and could be a useful tool when breeding for IMI resistance in sunflower. Nevertheless, further studies will be necessary to describe the mechanisms of resistance with more detail.

In conclusion, *in vivo* AHAS assay and the comparison of parameters from the dose–response curves were a valid tool for comparing IMI resistance in sunflower inbred lines and their hybrids. It was possible to discriminate homozygous from heterozygous genotypes for I_{mr1} *locus* that codify for the catalytic subunit of AHAS. Imazethapyr was found to be a stronger inhibitor than imazapyr, probably due to a differential metabolism by the plant.

The method described in this study was useful for the selection of sunflower genotypes differing in IMI resistance and could allow the implementation of a fast and reliable diagnosis method that could be helpful to assist breeding programs.

3. Materials and methods

3.1. Plant materials

Three sunflower inbred lines and their F_1 hybrids were used in this study. The inbred lines evaluated were: HA425, 1058-1 and HA89 which are IMI resistant, intermediate and susceptible, respectively. The resistant line HA425 is a BC_2F_6 maintainer germplasm selected from the cross HA89*3/PUR *H. annuus*. This line was developed and released by the USDA-ARS and the North Dakota Agricultural Experiment Station [19]. The line 1058-1 was developed from a backcross between HA425 and HA89 [39]. The F_1 hybrids among these inbred lines were generated at the Campo Experimental J. Villarino, Zavalla, Argentina during summer 2007–2008. Table 1 summarizes the plant materials used in this study. Achenes were planted on plastic pots (4 cm wide, 5.5 cm tall) filled with commercial perlite and watered by capillarity with nutritive solution consisting of Murashige and Skoog's [40] medium (25% v/v). Pots were incubated in growth chamber under controlled conditions (25 ± 2 °C, 12 h light).

3.2. In vivo AHAS assay

AHAS activity was determined by the procedure of Gerwick et al. with minor modifications as described below [27,41]. Youngest leaves (150–200 mg) of V2-stage plants [42] were used. A stock incubation

Table 1
Inbred lines and hybrids used in this study with their correspondent phenotypic and genotypic classification according to Bruniard and Miller [17].

Plant material	Genotype	Phenotype
Inbred lines		
HA425	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$	IMI resistant
1058-1	$I_{mr1}I_{mr1}i_{mr2}i_{mr2}$	IMI intermediate
HA89	$i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	IMI susceptible
F1 hybrids		
HA425 × 1058-1	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$	IMI resistant
HA425 × HA89	$I_{mr1}i_{mr1}I_{mr2}i_{mr2}$	IMI intermediate
HA89 × 1058-1	$I_{mr1}i_{mr1}i_{mr2}i_{mr2}$	IMI intermediate

Table 2
In vivo acetohydroxyacid synthase (AHAS) parameters estimates and standard errors for resistant (R), intermediate (I), susceptible (S) lines and their *F*1 hybrids.

Genotype		b[a]		e[b]	
		Imazapyr	Imazethapyr	Imazapyr	Imazethapyr
R	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$	0.61 ± 0.07	0.54 ± 0.08	85.3 ± 19.0	89.1 ± 24.7
I	$I_{mr1}I_{mr1}i_{mr2}i_{mr2}$	0.51 ± 0.06	0.56 ± 0.10	71.8 ± 18.6	56.9 ± 20.5
S	$i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	1.77 ± 0.29	1.0 ± 0.2	5.1 ± 0.5	0.9 ± 0.2
R × I	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$	0.89 ± 0.18	1.2 ± 0.2	133.5 ± 28.1	73.7 ± 11.3
R × S	$I_{mr1}i_{mr1}I_{mr2}i_{mr2}$	0.60 ± 0.06	0.51 ± 0.08	24.5 ± 5.1	5.6 ± 2.3
S × I	$I_{mr1}i_{mr1}i_{mr2}i_{mr2}$	0.53 ± 0.06	0.57 ± 0.08	17.9 ± 4.4	12.9 ± 4.6

[a] Slope at inflection point of the sigmoid curve.
[b] Herbicide dose in μM that reduced 50% *in vivo* activity of AHAS compared with the untreated control.

solution containing 25% (v/v) of Murashige and Skoog's [40] medium, 0.025% Triton X-100 and 500 μM 1,1-cyclopropanedicarboxylic acid (CPCA) was prepared immediately before each assay. Imidazolinone herbicide, imazapyr or imazethapyr, was added to a subsample of the incubation solution and serial dilutions were performed to achieve doses of 0.1, 1, 3.16, 10, 31.6, 100, 316 and 1000 μM. For each dose, positive control (incubation solution without herbicide) and herbicide treatment were made on opposite leaves from the same plant. Petri dishes, containing leaf samples and 6 ml of incubation solution, were placed in a growth chamber at 25 °C under fluorescent light (100 $\mu mol/m^2/s^1$) for 6 h. After incubation, leaves were weighted and stored at −20 °C until used. Leaf tissue was grounded in liquid nitrogen with 1 ml of water per gram of fresh weight and 100 mg of polyvinylpolypyrrolidone (PVPP). The samples were incubated at 60 °C for 15 min followed by incubation at room temperature for 45 min, with vortexing every 15 min to facilitate 2-acetolactate extraction from leaf tissues. After centrifugation at 4 °C for 10 min (11,200 *g*) a 400-ml aliquot was taken and mixed with 40 ml of 5.5 N H_2SO_4. The mixture was incubated at 60 °C for 30 min to facilitate decarboxilation. Acetoin and 3-hydroxy-2-pentanone were quantified by a modified colorimetric assay [29] in which the color was developed by adding 350 μl of 0.5% (w/v) creatine and 350 μl of 5% (w/v) 1-naphthol prepared in 2 N NaOH just before use. The samples were vortexed, incubated at 60 °C for 15 min, allowed to cool and centrifuged at 25 °C for 5 min (11,200 *g*). Absorbance of the 2,3-diketone species was measured spectrophotometrically at 530 nm [43]. Acetoin-forming enzymes in plant tissues [44] may interfere with the assay, thus the contribution of the direct formation of acetoin by non-AHAS enzyme activities was determined using NaOH to terminate the reaction instead of H_2SO_4 [45]. For each herbicide dose, AHAS activity was calculated as a percentage of absorbance in relation to the corresponding positive control.

3.3. Statistical analysis

An experiment for each herbicide (imazapyr and imazethapyr) was conducted in a completely randomized design with three

Table 3
Pairwise comparisons between *in vivo* acetohydroxyacid synthase (AHAS) parameters for resistant (R), intermediate (I), susceptible (S) inbred lines and their *F*1 hybrids (R × I, R × S and S × I).

Comparison	Genotypes	b[a]		I_{50}[b]	
		Imazapyr	Imazethapyr	Imazapyr	Imazethapyr
R vs R × I	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $I_{mr1}I_{mr1}I_{mr2}i_{mr2}$	ns[c]	$p < 0.05$	ns	ns
R vs I	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $I_{mr1}I_{mr1}i_{mr2}i_{mr2}$	ns	ns	ns	ns
R × I vs I	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$ vs $I_{mr1}I_{mr1}i_{mr2}i_{mr2}$	$p < 0.05$	$p < 0.05$	ns	ns
R × I vs R × S	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$ vs $I_{mr1}i_{mr1}I_{mr2}i_{mr2}$	ns	$p < 0.01$	$p < 0.001$	$p < 0.01$
I vs S × I	$I_{mr1}I_{mr1}i_{mr2}i_{mr2}$ vs $I_{mr1}i_{mr1}i_{mr2}i_{mr2}$	ns	ns	$p < 0.01$	$p < 0.05$
R × S vs S × I	$I_{mr1}i_{mr1}I_{mr2}i_{mr2}$ vs $I_{mr1}i_{mr1}i_{mr2}i_{mr2}$	ns	ns	ns	ns
R vs S	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	$p < 0.001$	$p < 0.05$	$p < 0.001$	$p < 0.05$

Pairwise comparisons for the *in vivo* acetohydroxyacid synthase (AHAS) parameters for the sunflower materials with the same genetic constitution at *locus* Imr_1.

Comparison	Genotypes		b[a]		I_{50}[b]	
	I_{mr1} *locus*	I_{mr2} *locus*	Imazapyr	Imazethapyr	Imazapyr	Imazethapyr
R vs R × I	$I_{mr1}I_{mr1}$	$I_{mr2}I_{mr2}$ vs $I_{mr2}i_{mr2}$	ns	$p < 0.05$	ns	ns
R vs I	$I_{mr1}I_{mr1}$	$I_{mr2}I_{mr2}$ vs $i_{mr2}i_{mr2}$	ns	ns	ns	ns
R × I vs I	$I_{mr1}I_{mr1}$	$I_{mr2}i_{mr2}$ vs $i_{mr2}i_{mr2}$	$p < 0.05$	$p < 0.05$	ns	ns
R × S vs S × I	$I_{mr1}i_{mr1}$	$I_{mr2}i_{mr2}$ vs $i_{mr2}i_{mr2}$	ns	ns	ns	ns

Pairwise comparisons for the *in vivo* acetohydroxyacid synthase (AHAS) parameters for the sunflower materials with the same genetic constitution at *locus* Imr_2.

Comparison	Genotypes		b[a]		I_{50}[b]	
	I_{mr1} *locus*	I_{mr2} *locus*	Imazapyr	Imazethapyr	Imazapyr	Imazethapyr
I vs S	$I_{mr1}I_{mr1}$ vs $i_{mr1}i_{mr1}$	$i_{mr2}i_{mr2}$	$p < 0.001$	$p < 0.05$	$p < 0.001$	$p < 0.01$
I vs S × I	$I_{mr1}I_{mr1}$ vs $I_{mr1}i_{mr1}$	$i_{mr2}i_{mr2}$	ns	ns	$p < 0.01$	$p < 0.05$
S × I vs S	$I_{mr1}i_{mr1}$ vs $i_{mr1}i_{mr1}$	$i_{mr2}i_{mr2}$	$p < 0.001$	ns	$p < 0.01$	$p < 0.05$
R × I vs R × S	$I_{mr1}I_{mr1}$ vs $I_{mr1}i_{mr1}$	$I_{mr2}i_{mr2}$	ns	$p < 0.01$	$p < 0.001$	$p < 0.001$

Pairwise comparisons for the *in vivo* acetohydroxyacid synthase (AHAS) parameters for the sunflower genotypes that differ at both *loci*.

Comparison	Genotypes	b[a]		I_{50}[b]	
		Imazapyr	Imazethapyr	Imazapyr	Imazethapyr
R vs S	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	$p < 0.001$	$p < 0.05$	$p < 0.001$	$p < 0.05$
R × I vs S × I	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$ vs $i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	ns	$p < 0.05$	$p < 0.001$	$p < 0.001$
R vs R × S	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $I_{mr1}i_{mr1}I_{mr2}i_{mr2}$	ns	ns	$p < 0.01$	$p < 0.01$
R vs S × I	$I_{mr1}I_{mr1}I_{mr2}I_{mr2}$ vs $I_{mr1}i_{mr1}i_{mr2}i_{mr2}$	ns	ns	$p < 0.001$	$p < 0.01$
I vs R × S	$I_{mr1}I_{mr1}i_{mr2}i_{mr2}$ vs $I_{mr1}i_{mr1}I_{mr2}i_{mr2}$	ns	ns	$p < 0.05$	$p < 0.05$
R × I vs S	$I_{mr1}I_{mr1}I_{mr2}i_{mr2}$ vs $i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	$p < 0.05$	ns	$p < 0.001$	$p < 0.001$
R × S vs S	$I_{mr1}i_{mr1}I_{mr2}i_{mr2}$ vs $i_{mr1}i_{mr1}i_{mr2}i_{mr2}$	$p < 0.001$	ns	$p < 0.001$	$p < 0.05$

[a] Slope at inflection point of the sigmoid curve.
[b] Herbicide dose in μM that reduced 50% *in vivo* activity of AHAS compared with the untreated control.
[c] ns: Non-significant.

replications. Data were analyzed using a nonlinear regression model [46]. Multiple dose–response curves were generated and analyzed using *R* statistical software [47,48]. The quality of each set of dose–response models was compared with an ANOVA by a lack-of-fit *F*-test [49]. The relationship between herbicide dose and AHAS activity was described using the three-parameter log–logistic model:

$$f(x) = d/\{1 + \exp(b(\log(x) - \log(e)))\}$$

where e (also known I_{50}) denotes the herbicide dose that inhibited AHAS activity by 50%; d reflects the response upper limit and b denotes the relative slope around e. The response lower limit is considered equal to 0. Testing for similarity of parameters such as e and b was done by comparing the fit of multiple dose–response models by an *F*-test based on the residual sum of squares of the two models. This test compares parameters values of interest among curves in order to detect statistically significant differences among the curves [48,49].

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