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ORIGINAL PAPER

Response to imazapyr and dominance relationships of two imidazolinone-tolerant alleles at the *Ahasl1* locus of sunflower

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Abstract Imisun and CLPlus are two imidazolinone (IMI) tolerance traits in sunflower (*Helianthus annuus* L.) determined by the expression of different alleles at the same locus, *Ahasl1-1* and *Ahasl1-3*, respectively. This paper reports the level of tolerance expressed by plants containing both alleles in a homozygous, heterozygous and in a heterozygous stacked state to increasing doses of IMI at the enzyme and whole plant levels. Six genotypes of the *Ahasl1* gene were compared with each other in three different genetic backgrounds. These materials were treated at the V2–V4 stage with increasing doses of imazapyr (from 0 to 480 g a.i. ha^{-1}) followed by an assessment of the aboveground biomass and herbicide phytotoxicity. The estimated dose of imazapyr required to reduce biomass accumulation by 50% (GR_{50}) differed statistically for the six genotypes of the *Ahasl1* gene. Homozygous CLPlus (*Ahasl1-3/Ahasl1-3*) genotypes and materials containing a combination of both tolerant alleles (Imisun/CLPlus heterozygous stack, *Ahasl1-1/Ahasl1-3*) showed the highest values of GR_{50}, 300 times higher than the susceptible genotypes and more than 2.5 times higher than homozygous Imisun materials (*Ahasl1-1/Ahasl1-1*). In vitro AHAS enzyme activity assays using increasing doses of herbicide (from 0 to 100 μM) showed similar trends, where homozygous CLPlus materials and those containing heterozygous stacks of Imisun/CLPlus were statistically similar and showed the least level of inhibition of enzyme activity to increasing doses of herbicide. The degree of dominance for the accumulation of biomass after herbicide application calculated for the *Ahasl1-1* allele indicated that it is co-dominant to recessive depending on the imazapyr dose used. By the contrary, the *Ahasl1-3* allele showed dominance to semi dominance according to the applied dose. This last allele is dominant over *Ahasl1-1* over the entire range of herbicide rates tested. At the level of enzymatic activity, however, both alleles showed recessivity to semi-recessivity with respect to the wild-type allele, even though the *Ahasl1-3* allele is dominant over *Ahasl1-1* at all the herbicides rates used.

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Introduction

The imidazolinone family of herbicides control weeds by inhibiting a key enzyme in the branched chain amino acid biosynthetic pathway, acetohydroxyacid synthase (AHAS; EC 4.1.3.18) also known as acetolactate synthase (ALS) (Shaner et al. 1984; Tan et al. 2005). A variety of crops tolerant to AHAS-inhibiting herbicides, such as corn (*Zea mays* L.), canola (*Brassica napus* L.), sugarbeet (*Beta vulgaris* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), sunflower (*Helianthus annuus* L.), flax (*Linum usitatissimum* L.), soybean [*Glycine max* (L.) Merr.] and wheat (*Triticum aestivum* L.), have been developed by a variety of approaches including somatic cell selection, mutation breeding, genetic modification and interspecific hybridization (Anderson and Georgeson 1989; Croughan 1996; D'Halluin et al. 1992; Newhouse et al. 1991; Hart et al. 1992; Wright and Penner 1998; Swanson et al. 1989; Subramanian et al. 1990; Rajasekaran et al. 1996; Sebastian et al. 1989; Pozniak and Hucl 2004; Al-Khatib and Miller 2000; Mallory-Smith et al. 1990; McHughen 1989). In most

of these cases, tolerance is due to a form of AHAS enzyme that is less sensitive to herbicide inhibition due to reduced herbicide binding. This reduction in herbicide binding is caused by mutations at key sites in the genes coding for the catalytic subunit of AHAS. Several authors have reviewed known mutations of the AHAS genes that confer tolerance to AHAS-inhibiting herbicides in plants (Preston and Mallory-Smith 2001; Tranel and Wright 2002; Tan et al. 2005). No known amino acid substitutions in the regulatory subunit have been reported to confer herbicide tolerance.

Based on molecular studies, Kolkman et al. (2004) identified and characterized three genes coding for the AHAS catalytic subunits in sunflower (*Ahasl1*, *Ahasl2* and *Ahasl3*). *Ahasl1* is a multilallelic locus and, so far, is the only member of this small family where all induced and natural mutations for herbicide tolerance in sunflower have been discovered. *Ahasl1-1* (also known as *Imr1* or Ar_{pur}, Bruniard and Miller 2001; Kolkman et al. 2004; respectively) harbors a C-to-T mutation in codon 205 (relative to *Arabidopsis thaliana* nomenclature) which confers a moderate tolerance to imidazolinones, *Ahasl1-2* (also known as Ar_{kan}) shows a C-to-T mutation in codon 197 conferring high levels of sulfonylurea tolerance (Kolkman et al. 2004), and *Ahasl1-3* has a G-to-A mutation in codon 122 which confers high levels of tolerance to imidazolinones (Sala et al. 2008b). Both *Ahasl1-1* and *Ahasl1-3* alleles are being used for the production of sunflower hybrids tolerant to imidazolinones.

The first commercial imidazolinone tolerance trait in sunflowers is known as 'Imisun' and its development started in 1996, when imidazolinone-tolerant wild sunflowers were discovered in a field in Kansas, USA. Subsequent crossing of these plants with cultivated sunflower lines gave rise to imidazolinone-tolerant populations and lines (Al-Khatib et al. 1998) which were released as donor materials for developing hybrid varieties. The first Imisun hybrid varieties were commercially launched in the USA, Argentina and Turkey in 2004.

The inheritance of Imisun is additively controlled by two components, where one of them is a partially dominant allele, *Ahasl1-1*, and the other, Imr_2, is a modifier or enhancer factor (Miller and Al-Khatib 2002; Bruniard and Miller 2001). To produce Imisun sunflower hybrids that express commercial tolerance levels to imidazolinone herbicides, both components need to be homozygous in the final variety. The second imidazolinone tolerance trait in sunflowers, known as CLPlus, is controlled by the expression of the partially dominant nuclear allele *Ahasl1-3* which was developed by seed mutagenesis and selection with imazapyr (Sala et al. 2008a). To achieve commercial tolerance levels in CLPlus sunflower hybrids, only one homozygous component, namely *Ahasl1-3*, is needed due to the high levels of imidazolinone tolerance conferred by this allele (Sala et al. 2008c) To date, both *Ahasl1-1* and *Ahasl1-3* alleles have been characterized as being partially dominant based on inheritance studies using only one or two doses of herbicide. However, studies in other plant species have shown that the dominance level in the presence of a herbicide can vary from completely dominant to completely recessive, depending on the tolerance allele and on the type and rate of herbicide tested (Roux et al. 2005).

Multiallelism at the *Ahasl1* locus of sunflower can be used to combine different herbicide-tolerant alleles in order to design new or specific tolerance traits in the commercial F_1 hybrid. For this reason, the objectives of this work were (1) to quantify the response to different doses of imazapyr at the whole plant and enzymatic levels in sunflower genotypes carrying different combinations of the imidazolinone-tolerant alleles, and (2) to determine the dominance relationships between these alleles.

Materials and methods

Plant material

Six different genotypes for the *Ahasl1* locus were assessed in three different genetic backgrounds (Table 1). Susceptible genotypes included a maintainer line (BTK47), a commercial restorer line (R20) and its F_1 hybrid (cmsBTK47/R20). CLPlus tolerant genotypes included GM40, R720 and its F_1 hybrid (H3). GM40 is the original mutant line from BTK47 which carries the *Ahasl1-3* mutation in a homozygous state (Sala et al. 2008b). R720 is a BC_3F_4 restorer line obtained by converting R20 to the CLPlus trait using GM40 as a donor line. IMISUN tolerant genotypes included IB9, IR7 and its F_1 hybrid H2. IB9 traces back to BTK47 and IR7 to R20. IMISUN heterozygous genotypes included H3 (cmsIB9/BTK47), H4 (cmsIB9/R20) and H5 (cmsBTK47/IR7). CLPlus heterozygous materials included H7 (cmsGM40/BTK47), H8 (cmsGM40/R20) and H9 (cmsBTK47/R720). Finally, Imisun/CLPlus heterozygous stacks included three F1 hybrids: H10 (cmsGM40/IB9), H11 (cmsGM40/IR7) and H12 (cmsIB9/R720).

Seeds of each genotype were sown in Petri dishes; after germination, seedlings were transplanted into potting media consisting of equal parts of vermiculite, soil and sand in 10 cm diameter pots. Plants were grown in a greenhouse under natural light conditions supplemented with 400 W sodium halide lamps to provide a 16 h photoperiod. Day/night temperatures were 25 and 20°C, respectively. At the V2–V4 stage (Schneiter and Miller 1981) 10 plants of each genotype were randomly assigned to each treatment consisting of seven doses of imazapyr

Table 1 Genotype for the *Ahasl1* locus, tolerance to imidazolinone, type of the trait, reproductive group and pedigree information for the lines and hybrids used in the dose–response experiment

Sunflower line or hybrid	Reproductive group	Pedigree or origin	*Ahasl1* Genotype	IMI tolerance	Name of the trait
BTK47	Maintainer	–	*ahasl1/ahasl1*	Susceptible	–
R20	Restorer	–	*ahasl1/ahasl1*	Susceptible	–
H1	Hybrid	BTK47/R20	*ahasl1/ahasl1*	Susceptible	–
IB9	Maintainer	–	*Ahasl1-1/Ahasl1-1*	Tolerant	Imisun homozygous
IR7	Restorer	–	*Ahasl1-1/Ahasl1-1*	Tolerant	Imisun homozygous
H2	Hybrid	IB9/IR7	*Ahasl1-1/Ahasl1-1*	Tolerant	Imisun homozygous
GM40	Maintainer	BTK 47 mutant	*Ahasl1-3/Ahasl1-3*	Tolerant	CLPlus homozygous
R720	Restorer	R20 conversion	*Ahasl1-3/Ahasl1-3*	Tolerant	CLPlus homozygous
H3	Hybrid	GM40/R720	*Ahasl1-3/Ahasl1-3*	Tolerant	CLPlus homozygous
H4	Hybrid	IB9/BTK47	*Ahasl1-1/ahasl1*	Tolerant	Imisun heterozygous
H5	Hybrid	IB9/R20	*Ahasl1-1/ahasl1*	Tolerant	Imisun heterozygous
H6	Hybrid	BTK47/IR7	*Ahasl1-1/ahasl1*	Tolerant	Imisun heterozygous
H7	Hybrid	GM40/BTK47	*Ahasl1-3/ahasl1*	Tolerant	CLPlus heterozygous
H8	Hybrid	GM40/R20	*Ahasl1-3/ahasl1*	Tolerant	CLPlus heterozygous
H9	Hybrid	BTK47/R720	*Ahasl1-3/ahasl1*	Tolerant	CLPlus heterozygous
H10	Hybrid	GM40/IB9	*Ahasl1-3/Ahasl1-1*	Tolerant	CLPlus/Imisun
H11	Hybrid	GM40/IR7	*Ahasl1-3/Ahasl1-1*	Tolerant	CLPlus/Imisun
H12	Hybrid	IB9/R720	*Ahasl1-3/Ahasl1-1*	Tolerant	CLPlus/Imisun

(0, 40, 80, 160, 240, 320, 400 and 480 g a.i. ha^{-1} which corresponded to 0.5×, 1×, 2×, 3×, 4×, 5× and 6× field rates, respectively) and subjected to the first (time-zero) biomass determination. The experiment was arranged as a randomized block design with a full factorial (sunflower line × treatment) arrangement of treatments in 10 replications.

On the day of herbicide application ten plants of each genotype were cut at the cotyledonal node and dried at 60°C for 48 h for the time-zero dried weight determination. The remaining plants were maintained for 14 days after imazapyr treatment (DAT) at which time the Phytotoxicity Index (PI) and above ground dry biomass were recorded. The above ground biomass data from each line was converted to biomass accumulation following application by subtracting the appropriate average time-zero biomass from each sample. Dry biomass data were converted to percentages of the untreated control plants within each line to allow direct comparisons between groups. The PI is a phenotypic scale from 0 to 9 where each plant is assessed visually for crop injury or phytotoxicity. Plants without any symptoms were recorded as "0"; plants with increasing levels of stunting and yellow coloration with respect to the untreated control plants were recorded as "1" to "4"; plants with increasing levels of leaf abnormalities and leaf necrosis were recorded from "5" to "8", and dead plants with complete necrosis of the apex were recorded as "9".

Statistical analysis of dose–response curves followed the procedure outlined by Seefeldt et al. (1995). Data were fit to a log-logistic model given by:

$$y = c + (d - c) \Big/ \left[1 + (x/\mathrm{GR}_{50})^{b}\right]$$

where y = shoot biomass (expressed as the percent of the untreated control), x = imazapyr dose (g a.i. ha^{-1}), c = lower asymptote, b is a rate parameter (slope) related to the response to increasing imazapyr dose, and GR_{50} is the imazapyr dose that caused a 50% of reduction in shoot biomass accumulation. Regressions were performed on all data using nonlinear least square regression procedure (PROC NLIN, SAS 2004). Adequacy of model fit was determined by significance of the model approximate F-statistic and the coefficients of determination. Comparisons of the regression parameters among the six genotypes for the *Ahasl1* locus were conducted by a nested analysis of variance using the model: y = genotype for the *Ahasl1* locus + genetic background (genotype for the *Ahasl1* locus) + error. Means were separated using Fisher's protected least significant difference (LSD) test at the 1 and 5% level of probability.

Enzyme assay for AHAS activity

In vitro assays of AHAS were conducted in two materials of each of the six genotypes for the *Ahasl1* locus to

determine the sensitivity of each of them at the AHAS level when challenged to increasing doses of imazapyr.

An assay measuring the level of inhibition of AHAS activity was performed on actively growing young leaves approximately 4 weeks after planting. The protein extraction was performed as follows. Leaves were ground under liquid N_2 and extracted with a buffer composed of 100 mM pyruvate, 200 mM KH_2PO_4, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate and 20 μM flavin adenine dinucleotide. The homogenate was filtered through two layers of Miracloth (Calbiochem) into a 50 ml conical polypropylene tube, followed by a step to remove leaf polyphenols. Approximately 4–5 ml of homogenate was then added to a prechilled and equilibrated Zeba Desalting Spin column and the column was centrifuged cold for 2 min at 1,000 rcf to obtain flowthrough. Flowthrough samples were assayed immediately. Using the Bradford protein assay (Bradford 1976), known amounts of bovine serum albumin (BSA) were used as a standard to estimate the amount of protein added to each reaction. The enzyme inhibition assay was performed essentially as described by Singh et al. (1988). Assays were performed in a 96-well format, with 50 μL of inhibitor per well plus 50 μL of soluble protein extract to give final concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μM imazapyr. For the negative control wells 20 μL of 5% H_2SO_4 was added. To stop the reaction, samples were place at 37°C for 2 h followed by 20 μL of 5% H_2SO_4 which was added to each well and the plate was incubated at 60°C for 15 min. For color development, 200 μL of a solution containing creatine (2.5 mg/ml) and α-naphthol (25 mg/ml) were added to each well. Plates were incubated at 60°C for 15 min and then removed and cooled for approximately 5–10 min. Absorbance was measured at 530 nm. Absorbance values for each treatment were expressed as AHAS activity (estimated by absorbance) and were calculated as a percentage of the mean of the zero-herbicide controls. Data from each line were fit to a nonlinear regression model by PROC NLIN of SAS (SAS Institute Inc, Cary NC, USA). The nonlinear regression was based on a logistic function mathematically described by Seefeldt et al. (1995).

AHAS activity (% of the mean of the zero herbicide controls) = $\beta_o + (\beta_1 - \beta_o)/[1 + (\text{dose}/I_{50})^{\beta 3}]$.

where β_o represents the lower asymptote of AHAS activity (%); β_1 represents the mean AHAS activity (%) in the zero-herbicide controls (i.e., upper asymptote); I_{50} represents the dose corresponding to AHAS activity midway between the upper and lower asymptotes (50% response);and β_3 (AHAS activity (%) dose^{-1}) represents the slope of the curve around the I_{50}. Dose represents the concentration of inhibitor used in the enzyme assay. Statistical analysis of AHAS inhibition curves and comparison of regression parameters among the six genotypes for the *Ahasl1* locus were conducted as described previously.

Dominance of herbicide tolerance

The dominance of herbicide tolerance was estimated for each dose of imazapyr treatment for biomass and for enzyme activity and was calculated using the formula: $D = (RS - SS)/(RR - SS)$ (Falconer 1964; Bourguet et al. 2000); where RR, RS and SS represent the phenotype of homozygous resistant, the heterozygous and the homozygous susceptible genotypes at each herbicide concentration. Thus, when RS = RR, $D = 1$ and the resistant phenotype is considered dominant. When RS = SS, $D = 0$ and the resistant phenotype is considered recessive. Finally, when RS presents an intermediate phenotype, $D = 0.5$ and alleles R and S are considered co-dominant. Five categories were defined according to Georghiou et al. (1966) and Bourguet and Raymond (1998): (1) Recessive, when $D \approx 0$; (2) Semi-recessive, when $0 < D < 0.5$; (2) Co-dominant, when $D \approx 0.5$; (4) Semi-dominant, when $0.5 < D < 1$; and (5) Dominant when $D \approx 1$.

Graphics showing the variation of the degree of dominance of the alleles *Ahasl1-1* and *Ahasl1-3* over the wild-type allele and between them as a function of herbicide doses were constructed for the biomass accumulation and for the enzymatic activity.

Results

The evaluated six genotypes showed significant differences for their tolerance to foliar imazapyr application as described below.

Phytotoxicity index

Both tolerant alleles, *Ahasl1-1* (Imisun) and *Ahasl1-3* (CLPlus), showed different levels of injury as the herbicide rate increased from 40 to 480 g a.i. ha^{-1} (Fig. 1). CLPlus plants carrying the *Ahasl1-3* allele in a homozygous state did not show significant differences with respect to control plants without herbicide application up to a 240 g a.i. ha^{-1} rate. As the rate increased, they only showed a slight reduction in leaf size and a lighter green color than the control plants as demonstrated by a PI value of 1.2 at the maximum rate of herbicide application (Table 2). In contrast, Imisun genotypes carrying the *Ahasl1-1* allele in a homozygous state did not show any symptoms at 40 or 80 g a.i. ha^{-1} of herbicide rate, but the level of injury (yellow leaf discoloration, leaf deformation and leaf necrosis) increased quickly from 160 to 480 g a.i. ha^{-1}. This was demonstrated by the PI values of 2.2 to 8.2, respectively (Table 2). The differences between both mutants in a homozygous state were significant for all the herbicide rates tested (Table 2). Heterozygous genotypes

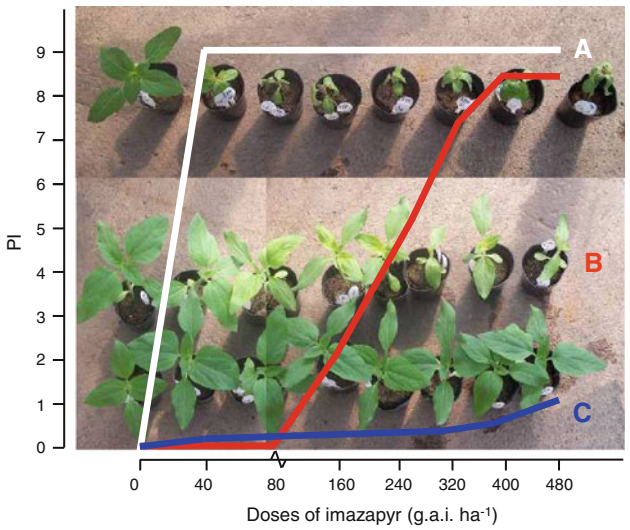

Fig. 1 Phenotype of representative plants of three genotypes at the *Ahasl1* locus (**a** wild type, **b** Imisun homozygote, and **c** CLPlus homozygote) 14 days after the application of different doses of imazapyr. *Curves* represent the mean phytotoxicity index (PI) of each type of material

Ahasl1-1/Ahasl1.3 showed the same pattern of response as the homozygous *Ahasl1-3* lines and hybrids. In fact, heterozygous *Ahasl1-1/Ahasl1-3* materials demonstrated only a slightly lighter green color than the control plants at any rate of herbicide application and a smaller leaf size than the control plants at 400 and 480 g a.i. ha^{-1} rates. For both of these latter rates, these stacked heterozygous genotypes demonstrated a PI of up to 1 at the higher doses (Fig. 1). In fact, there were no significant differences for the PI values between *Ahasl1-3* homozygotes and *Ahasl1-1/Ahasl1-3* heterozygotes at any rate of herbicide application (Table 2).

Heterozygotes carrying the *Ahasl1-1* showed PI values from 0.4 to 9 when challenged with 40 to 480 g a.i. ha^{-1} rates of imazapyr, respectively. In contrast, heterozygous CLPlus genotypes presented a PI of 0.8 to 5.7 when the same rates were applied (Table 2).

Biomass accumulation

Dose–response curves for the six analyzed genotypes based on dry weight are shown in Fig. 2. Susceptible materials, on average, showed a significant reduction in dry biomass weight 14 days after herbicide application at all rates of imazapyr, even at the lowest rate. Biomass reduction of the susceptible materials, when compared to the untreated control plants, was from 77.2 to 87.4% for the 40 and 480 g a.i. ha^{-1} rates, respectively (Table 3).

Dry weight of Imisun plants (*Ahasl1-1*) in a homozygous condition was significantly reduced with respect to the control plants from 14.6%, at the 80 g a.i. ha^{-1} rate, to 73.9%, at the 480 g a.i. ha^{-1} rate. Genetic materials carrying the *Ahasl1-1* allele in a heterozygous state showed the same pattern of reduction in dry weight after herbicide treatment from 34.8%, at the lowest rate, to 83.1%, at the highest rate tested.

Meanwhile, the biomass of homozygous CLPlus plants was not reduced significantly from 40 to 160 g a.i. ha^{-1}. At the 240 g a.i. ha^{-1} rate of herbicide application, dry weight of this genotype was reduced 10% with respect to control plants, and at the highest rate this reduction reached 30.1%. Genotypes carrying the *Ahasl1-3* allele mutation in heterozygous state showed a significant reduction in dry weight of 13.9% at 80 g a.i. ha^{-1} to 52.5% at the 480 g a.i. ha^{-1} rate of herbicide application, respectively.

Heterozygous *Ahasl1-1/Ahasl1-3* genotypes showed the same trend as homozygous *Ahasl1-3* materials, presenting a reduction in biomass weight from 6.7 to 39.2% for 160 to 480 g a.i. ha^{-1}. In fact, there were no significant differences between homozygous *Ahasl1-3* and heterozygous *Ahasl1-1/Ahasl1-3* genotypes with respect to this variable at any doses (Table 3). Both *Ahasl1-3* and *Ahasl1-1* alleles showed significant differences between each other with respect to the reduction in biomass weight from 80 to 480 g a.i. ha^{-1} in the case of homozygotes and from 40 to 480 g a.i. ha^{-1} in the case of heterozygotes (Table 3).

The log-logistic model accurately described biomass accumulation after imazapyr application for susceptible and tolerant sunflower plants (Fig. 2). Estimates of the doses of imazapyr needed to reduce the biomass accumulation of each genotype by the half (GR_{50}) varied from 1.9 to 658 g a.i. ha^{-1}, and were statistically different among the six genotypes evaluated (Table 4). Biomass accumulation of the susceptible materials was reduced to 50% with a dose of 1.9 g a.i. ha^{-1} of imazapyr, which represent only 2.4% of the 80 g a.i. ha^{-1} recommended as the rate under field conditions (1× rate). In contrast, CLPlus homozygous genotypes and those combining both tolerant alleles (*Ahasl1-1* and *Ahasl1-3*) showed the highest values of GR_{50}, more than 300 times greater than the susceptible genotypes and 2.5 times greater than the homozygous Imisun materials (Table 4). On the other hand, GR_{50} estimate for the Imisun genotypes was 233 g a.i. ha^{-1}, a dose which corresponds to a 3× application rate under field conditions. The behavior of the heterozygous genotypes was highly different from each other. The CLPlus heterozygotes tested showed a level of biomass accumulation 263 times greater than the susceptible checks and a GR_{50} value of 75% of that showed by their respective homozygous CLPlus genotypes. In contrast, heterozygous Imisun genotypes presented 35 times greater biomass accumulation than the susceptible checks, a value which represents only 28% of the GR_{50} estimates of their Imisun homozygous counterparts (Table 4).

Table 2 Mean phytotoxicity index evaluated 14 days after treatment with imazapyr on three sunflower lines or hybrids for each of six genotypes at the *Ahasl1* locus of sunflower

Doses (g a.i. ha$^{-1)}$	Genotypes					
	Susceptible	Imisun homozygous	CLPlus homozygous	Imisun heterozygous	CLPlus heterozygous	CLPlus/Imisun
Ahasl1 Genotype	*ahasl1/ahasl1*	*Ahasl1-1/Ahasl1-1*	*Ahasl1-3/Ahasl1-3*	*ahasl1/Ahasl1-1*	*ahasl1/Ahasl1-3*	*Ahasl1-3/Ahasl1-1*
480	9.0 ± 0.0	8.2 ± 1.3	1.2 ± 1.2	9.0 ± 0.0	5.7 ± 0.2	0.5 ± 0.0
400	9.0 ± 0.0	8.2 ± 1.2	0.8 ± 1.0	9.0 ± 0.0	5.2 ± 0.2	0.6 ± 0.2
320	9.0 ± 0.0	7.4 ± 1.3	0.5 ± 0.5	9.0 ± 0.0	3.4 ± 0.7	0.5 ± 0.0
240	9.0 ± 0.0	5.1 ± 1.3	0.3 ± 0.3	8.9 ± 0.2	2.7 ± 1.1	0.5 ± 0.0
160	9.0 ± 0.0	2.2 ± 0.8	0.3 ± 0.3	8.0 ± 0.1	1.3 ± 0.4	0.5 ± 0.0
80	8.9 ± 0.1	0.0 ± 0.0	0.3 ± 0.3	5.8 ± 0.2	1.0 ± 0.0	0.5 ± 0.0
40	8.7 ± 0.3	0.0 ± 0.0	0.3 ± 0.3	0.4 ± 0.2	0.8 ± 0.3	0.5 ± 0.0
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Plants without any symptoms were recorded as "0", increasing levels of stunting and yellowing with respect to the untreated control plants were recorded as "1" to "4"; increasing levels of leaf abnormalities and leaf necrosis were recorded from "5" to "8"; dead plants with total necrosis of the apex were recorded as "9"

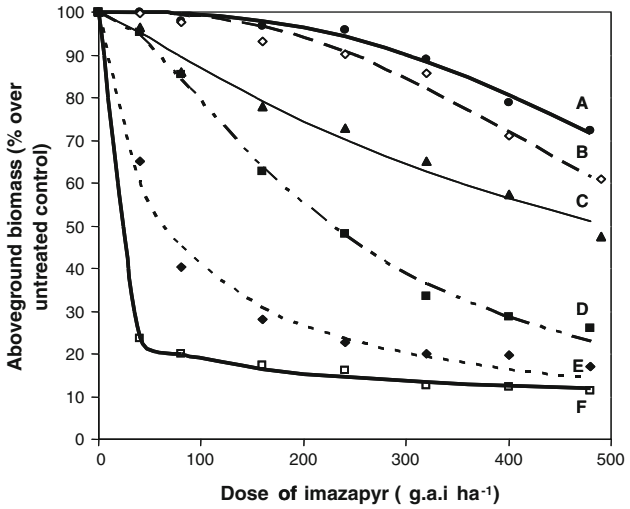

Fig. 2 Biomass accumulation 14 days after the application of different doses of imazapyr on three sunflower lines or hybrids for each of six genotypes at the *Ahasl1* locus of sunflower. **a** CLPlus homozygous (*Ahasl1-3/Ahasl1-3*), **b** CLPlus/Imisun (*Ahasl1-3/Ahasl1-1*), **c** CLPlus heterozygous (*Ahasl1-3/ahasl1*), **d** Imisun homozygous (*Ahasl1-1/Ahasl1-1*), **e** Imisun heterozygous (*Ahasl1-1/ahasl1*), **f** Susceptible (*ahasl1/ahasl1*)

AHAS activity

In vitro assays of AHAS activity were conducted on two genetic materials for each of the six genotypes at the *Ahasl1* locus to determine the sensitivity of each of them at the AHAS level when challenged to increasing doses of imazapyr.

In vitro inhibition curves of AHAS activity at different doses of imazapyr for the six genotypes at the Ahasl1 locus are shown in Fig. 3. The log-logistic model accurately described specific activities of AHAS for all the genotypes (Table 5). The average value for the parameter β_o which represents the lower asymptote of AHAS activity inhibition curves was 63.2% ± 5.7 for CLPlus homozygotes, 64.7% ± 9.7 for Imisun/CLPlus heterozygotes, 28.3% ± 1.6 for CLPlus heterozygotes, 28.2% ± 2.3 for Imisun homozygotes, and 6.5 ± 2.6 for the group of susceptible genotypes. Differences among groups of genotypes for this parameter were highly significant ($p < 0.001$) and segregated them in three well defined clusters: one with the highest level of inhibition, which included susceptible and Imisun heterozygous genotypes; the second with a moderate level of inhibition including Imisun homozygotes and CLPlus heterozygotes and the third with the lowest level of inhibition, which clustered together CLPlus homozygotes and CLPlus/Imisun heterozygotes (Table 5). These results indicate that much of the AHAS activity from CLPlus homozygotes and CLPlus/Imisun heterozygotes was insensitive to imazapyr when compared with the inhibition curve for the normal enzyme from the susceptible materials. Moreover, the AHAS activity of these materials doubled that obtained for the Imisun homozygotes and CLPlus heterozygotes.

Average estimated values for β_1 were almost the same for the six genotypes and were not different from 100% (Table 5). The values for the parameter I_{50} which represents the dose corresponding to AHAS activity midway between the upper and lower asymptotes were also different among groups of genotypes ($p < 0.001$). Since the lower asymptote of AHAS activity inhibition curves were different for all the genotypes, the estimates of I_{50} for them represent doses which correspond to different AHAS activities (82.2 and 53% of the untreated control plants for

Table 3 Mean biomass accumulation 14 days after treatment with imazapyr on three sunflower lines or hybrids for each of six genotypes at the *Ahasl1* locus of sunflower

Doses (g a.i. ha^{-1})	Genotypes						
	Susceptible	Imisun homozygous	CLPlus homozygous	Imisun heterozygous	CLPlus heterozygous	CLPlus/Imisun	LSD among genotypes
Ahasl1 Genotype	*ahasl1/ahasl1*	*Ahasl1-1/ Ahasl1-1*	*Ahasl1-3/ Ahasl1-3*	*ahasl1/ Ahasl1-1*	*ahasl1/ Ahasl1-3*	*Ahasl1-3/ Ahasl1-1*	
0	100.0 ± 0.0a*	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	
40	22.8 ± 4.3b	95.6 ± 3.8a	99.0 ± 2.1ab	65.2 ± 0.8b	96.3 ± 3.2a	99.6 ± 0.2a	7.3
80	19.5 ± 3.9bc	85.4 ± 4.0b	96.7 ± 2.6ab	38.8 ± 7.2c	86.1 ± 3.0b	97.6 ± 1.5a	12.3
160	17.3 ± 2.4cd	62.8 ± 11.2c	92.1 ± 9.2ab	28.1 ± 4.8d	77.8 ± 7.0c	93.3 ± 1.1b	13.4
240	16.5 ± 2.6cd	48.1 ± 12.4d	90.0 ± 12.4bc	22.7 ± 4.2e	72.9 ± 2.6c	90.1 ± 2.3c	12.6
320	12.8 ± 1.9d	33.5 ± 11.4e	85.7 ± 11.5c	19.1 ± 5.6ef	65.2 ± 1.7d	85.7 ±1.9d	12.5
400	12.9 ± 1.8d	28.7 ± 9.6e	74.2 ± 12.8d	19.3 ± 4.7ef	57.2 ± 3.4e	70.9 ± 1.9e	9.8
480	12.6 ± 2.1d	26.1 ± 7.3e	69.9 ± 10.0d	16.9 ± 4.2f	47.5 ± 1.4f	60.8 ± 2.2f	5.0
LSD among doses	4.7	9.1	9.7	4.9	5.6	2.5	

* Mean values with the same letter do not differ among doses

Table 4 Estimates of the doses of imazapyr needed to reduce the biomass accumulation by the half (GR_{50}), and tolerant (T)/susceptible (S) ratio estimated by nonlinear regression for biomass response to increasing doses of imazapyr of six genotypes at the *Ahasl1* locus

Genotype	GR_{50} (g a.i. ha^{-1})	GR_{50} ratio (T/S)
CLPlus homozygous	658.4a*	350
CLPlus/Imisun	573.2b	305
CLPlus heterozygous	493.9c	263
Imisun homozygous	233.2d	130
Imisun heterozygous	65.6e	35
Susceptible	1.9f	1
LSD	56.8	

* Mean values with the same letter do not differ among doses

CLPlus homozygotes and susceptible genotypes, respectively). In fact, the concentration of imazapyr needed to obtain 50% of inhibition of AHAS activity of the untreated control plants for the susceptible materials was about 6.5 μM, whereas for CLPlus homozygotes this percentage of inhibition was not observed even at the highest herbicide concentration (100 μM).

Dominance relationships

The degree of dominance for the accumulation of biomass calculated for the *Ahasl1-1* allele indicated that it is co-dominant ($D \approx 0.5$) to recessive ($D < 0.5$), depending on the imazapyr dose used. By the contrary, the *Ahasl1-3* allele showed dominance to semi dominance according to the applied dose. Moreover, this last allele is dominant over *Ahasl1-1* over the entire range of herbicide rates tested (Fig. 4).

At the level of enzymatic activity, however, both alleles showed recessivity to semi-recessivity with respect to the wild-type allele. On the other hand, the *Ahasl1-3* allele is dominant over *Ahasl1-1* at all the herbicides rates used (Fig. 3b).

Discussion

Imidazolinone is absorbed through both foliage and root tissues (Tu et al. 2001). After entering a plant, imidazolinone is transported through the xylem and phloem to meristematic tissues where it binds to AHAS and inhibits its activity. Structural analysis has shown that imidazolinone inhibits AHAS activity by blocking a channel leading to the active site, despite its structural dissimilarity from endogenous AHAS substrates (McCourt et al. 2006). Inhibition of AHAS leads to global elevation of free amino acids level and imbalances in their relative proportions (Höfgen et al. 1995); a relatively frequent outcome resulting from inhibition of an enzyme involved in amino acid biosynthesis pathways (Kim et al. 2002). In fact, time course analysis of transcriptome profiles in imidazolinone-sensitive (wild type) and imidazolinone-resistant genotypes of *Arabidopsis thaliana* has demonstrated that in wild-type plants, the genes which responded earliest to imazapyr treatment were detoxification-related genes. Later stages of the imazapyr response involved regulation of genes participating in biosynthesis of amino acids, secondary metabolites, and tRNA. In contrast, the transcriptome of

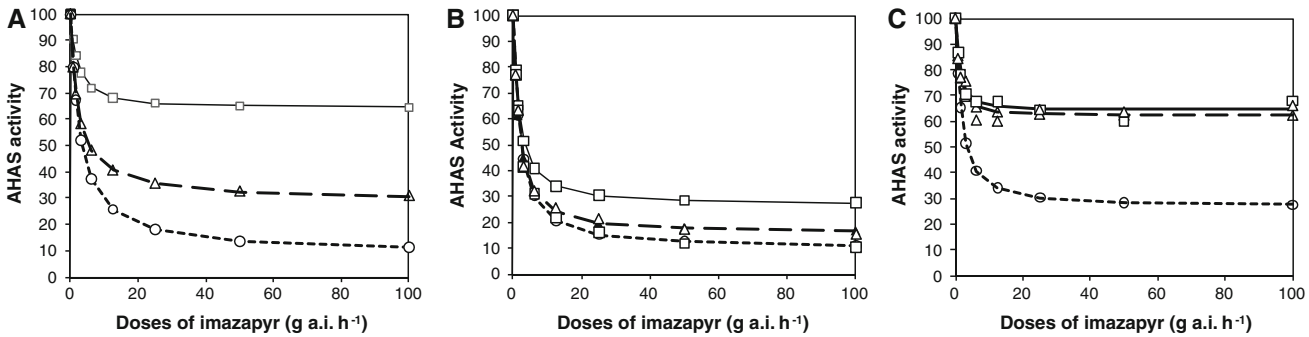

Fig. 3 In vitro inhibition curves of AHAS activity at different doses of imazapyr for different genotypes of at the sunflower *Ahasl1* locus. **a** Inhibition curves of AHAS activity for: Aa1: susceptible (*dotted lines*), CLPlus homozygous (*continuous lines*) and CLPlus heterozygous genotypes (*dashed lines*); a2B: Susceptible (*dotted lines*), Imisun homozygous (*continuous lines*) and Imisun heterozygous (*dashed lines*); a3C: Imisun homozygous (*dotted lines*), CLPlus homozygous (*continuous lines*) and the Imisun/CLPlus heterozygous (*dashed lines*). ****b Dominance relationships for different alleles of the *Ahasl1* locus at the enzymatic level. b1: *Ahasl1-3* (CLPlus allele), b2: *Ahasl1-1* (Imisun allele) and b3: *Ahasl1-3* over *Ahasl1-1*

Table 5 Estimates of the parameters of the equation describing the kinetics of in vitro AHAS activity inhibition by imazapyr of six genotypes at the *Ahasl1* locus of sunflower

Genotypes	β_o	β_1	I_{50}	β_3
Susceptible	6.45 ± 2.62c*	99.5 ± 0.14a	2.95 ± 0.21a	−0.965 ± 0.04a
Imisun homozygous	28.15 ± 2.33b	100 ± 0.00a	1.77 ± 0.47bc	−0.755 ± 0.06a
Imisun heterozygous	14.5 ± 0.99c	100 ± 0.00a	1.9 ± 0.11bc	−0.845 ± 0.02a
CLPlus homozygous	63.15 ± 5.73a	100.45 ± 0.64a	1.015 ± 0.08c	−1.81 ± 0.98a
CLPlus/Imisun	64.65 ± 9.65a	100.25 ± 0.07a	1.095 ± 0.09c	−1.41 ± 0.41a
CLPlus heterozygous	28.3 ± 1.56b	100 ± 0.00a	2.435 ± 0.74ab	−1.045 ± 0.15a

The regression equation is of the following form: AHAS activity (% of the mean of the zero herbicide controls) = $\beta_o + (\beta_1 - \beta_o)/[1+(\text{dose}/I_{50})^{\beta 3}]$, where β_o represents the lower asymptote of AHAS activity (%); β_1 represents the upper asymptote; I_{50} represents the dose corresponding to AHAS activity midway between the upper and lower asymptotes; and β_3 represents the slope of the curve around I_{50}. Mean values and their standard deviations of each of the four parameters of the equations are provided

* Mean values with the same letter do not differ among doses

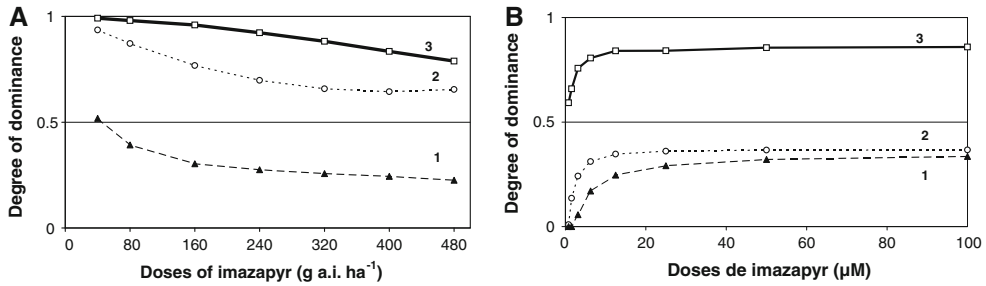

Fig. 4 Degree of dominance for two imidazolinone tolerance-conferring alleles of sunflower **a** For biomass accumulation 14 days after imazapyr application **b** For AHAS activity inhibition. *1* Dominance level of *Ahasl1-1* (Imisun allele) over the wild-type susceptible allele, *2* Dominance level of *Ahasl1-3* (CLPlus allele) over the wild-type susceptible allele. *3* Dominance level of *Ahasl1-3* over *Ahasl1-1*

resistant plants did not exhibit significant changes following imazapyr treatment. Thus, all of the changes caused by imazapyr treatment in susceptible plants, including global transcriptome expression, growth inhibition, and eventual plant death are all caused by the inhibition of AHAS function (Manabe et al. 2007).

Results of the quantitative imazapyr response in Imisun and CLPlus homozygous and heterozygous sunflower lines and hybrids can be interpreted by the relative tolerance levels of their respective AHAS enzymes and by the associated changes that would occur at the transcriptome level. A122T substitution in the *Ahasl1* gene displayed the

lowest level of inhibition of the AHAS enzyme extracts, which results in the higher level of accumulation of biomass at all rates of herbicide application. A205V substitution, on the other hand, showed a moderate level of tolerance and a higher inhibition of AHAS activity. As the inhibition of AHAS increase, the transcriptome of *Ahasl1-1/Ahasl1-1* plants would initiate significant changes, with the induction of several non-target metabolic pathways, like the expression of detoxification genes and secondary metabolites. These changes permit to explain the growth inhibition at the lower doses and the eventual plant death at the higher doses of the Imisun genotypes. In fact, the accumulation of biomass after 2 weeks of herbicide application is highly associated with in vitro enzyme activities when challenged with imazapyr. CLPlus homozygous and CLPlus/Imisun heterozygous genotypes showed the lowest level of inhibition and the highest biomass accumulation. Susceptible and Imisun heterozygous materials, on the other hand, showed the highest level of enzymatic inhibition and the lowest biomass accumulation. Between both extremes, Imisun homozygous and CLPlus heterozygous genotypes had the same intermediate level of enzymatic kinetics and a moderate level of biomass accumulation. Nevertheless, the biomass accumulation of the CLPlus heterozygous materials was higher than that observed in the Imisun homozygous genotypes.

Literature about herbicide tolerance indicates that almost all tolerances are inherited as partially to totally dominant traits (Gould 1995; Warwick 1991). However, most studies reporting the degree of dominance of an herbicide-tolerance trait were designed to assess the inheritance of the tolerance mutation. As a consequence, a single threshold herbicide is generally used and this single dose approach may not be appropriate to correctly assess dominance since the applied dose may affect apparent dominance and recessivity (Roux et al. 2005).

Using two alleles for imidazolinone tolerance we found that the dominance level in the presence of herbicide can vary from dominance to recessivity depending on the tolerance allele, the applied dose of herbicide and the variable considered (biomass accumulation or enzymatic activity). This dominance variation, at least for a phenotypic variable like root length, was also observed for the *csr1-1* and *csr1-2* alleles of *Arabidopsis thaliana* when challenged with six AHAS inhibitors (Roux et al. 2005).

At the enzymatic level both CLPlus and Imisun alleles showed a similar pattern of dominance relationship when challenged with increased doses of herbicide i.e.: from completely recessivity to semi-recessivity. This general pattern can be explained by taking into consideration the presence of sensitive and insensitive AHAS enzymes in the heterozygotes. In the absence of herbicides, heterozygous plants possess 100% of enzyme activity. When the concentration of imazapyr increases, sensitive enzymes are drastically inhibited until enzyme activity is only provided by insensitive targets. Dominance for enzyme activity of *Ahasl1-3* over *Ahasl1-1,* on the other hand, can be explained taking into account the protein structure of the AHAS catalytic subunits. The AHAS enzyme from plants is thought to be an assembly consisting of four catalytic and four regulatory subunits (Duggleby and Pang 2000; Duggleby et al. 2003, 2008). Each active site of the enzyme is at the interface of two monomers; hence the minimal requirement for AHAS activity is a dimmer of the catalytic subunits (McCourt et al. 2006). IMI herbicides inhibit AHAS by binding within and obstructing the channel leading to the active site of the enzyme. The 3D structure of AHAS allowed McCourt and co-workers (McCourt et al. 2006) to explain how several amino acid substitutions in the active site of the enzyme result in tolerance to imidazolinones. Hence, A122 substitutions make important hydrophobic contacts to the isopropyl and methyl substituents of the dihydroimidazolinone ring of the IMI molecule, and a mutation to a larger polar residue such as a threonine would tend to preclude the herbicide from its binding site, as in the CLPlus AHAS enzyme. Heterozygous individuals combining *Ahasl1-1* and *Ahasl1-3* alleles present three types of enzymes with respect to the conformation of the herbicide-binding site: two of them composed by identical subunits (i.e., A205V/A205V or A122T/A122T) and the third composed by different subunits (A205V/A122T). The dominance of the *Ahasl1-3* allele over the *Ahasl1-1* allele both at the molecular and phenotypic levels permits to speculate that the enzyme composed by both subunits (A205V/A122T) is as strong as the A122T enzyme with respect to its tolerance to imazapyr. CLPlus heterozygous genotypes and Imisun homozygotes presented the same enzyme inhibition kinetics but different levels of injury or biomass accumulation after herbicide application. This indicates that even though the AHAS inhibition values of a pool of homogeneous AHAS protein (like that presented by the Imisun genotypes) is similar to a heterogeneous pool of AHAS protein (CLPlus heterozygous genotypes), their biological relevance may not be same. In fact, in the case of CLPlus heterozygous genotypes the inhibition kinetics of AHAS is assessed on a pool of three different types of AHAS enzyme with respect to the conformation of the herbicide binding site: one composed by wild-type subunits, another composed by A122T subunits and the third one composed by a mix of both types of subunits. The kinetics of the inhibition reflects, on average, the behavior of these three types of enzymes. Since the wild-type portion of this pool is a sensitive target, the heterogeneous pool is drastically inhibited initially until only insensitive targets provide the AHAS activity.

Wright (1929) defined the margin of error for an enzyme as the maximum decrease of the enzyme activity that can be tolerated without affecting the phenotype. Kacser and Burns (1981) and Keightley and Kacser (1987) showed that this safety margin is the consequence of the kinetic structures of enzymatic pathways since enzyme activity is far in excess of that necessary. For this reason, a change in enzyme activity or concentration at any one step in a multistep metabolic pathway is unlikely to have a large effect on the output of the system. This safety margin was documented for enzyme targets of pesticides. For example, less than 30% of acetylcholinesterase wild-type activity is sufficient to ensure viability in several species of insects (Fournier and Mutéro 1994). Similarly, *Sacharomyces cerevisiae* mutants with only 10% of the wild-type AHAS activity are viable (Falco and Dumas 1985). This excess of enzyme activity would explain why the enzyme target modification conferring CLPlus imidazolinone tolerance is mostly semi-dominant at the phenotypic level, even though it is recessive at the enzymatic level. In addition, structural enzymatic changes caused by herbicide-tolerance mutations may result in either subtle or drastic modifications of substrate and/or inhibitor binding leading to insufficient (impaired) activity, imbalance (feedback inhibition) or excess (higher activity) of enzyme end-product biosynthesis (Yu et al. 2010) leading to reduced plant growth (Vila-Aiub et al. 2009). Therefore it is likely that some resistance-conferring mutations would impair AHAS functionality. Indeed, for other herbicides, it is known that resistance mutations reduce enzyme activity (e.g. for EPSPS mutations, Healy-Fried et al. 2007, for ACCase mutations, Yu et al. 2007b). However, the situation appeared to be more complex for AHAS. Depending on plant species and the particular AHAS amino acid substitution conferring tolerance, different results were reported showing reduced (Eberlein et al. 1997; Ashigh and Tardif 2007; Yu et al. 2010), increased (Boutsalis et al. 1999; Purrington and Bergelson 1999; Yu et al. 2007a, b) or unchanged (Boutsalis et al. 1999; Preston et al. 2006; Yu et al. 2010) AHAS activity. In fact, the individual impact of the known 22 resistance-endowing AHAS gene mutations on AHAS functionality and their concomitant effect on plant fitness remains unknown and needs empirical evaluation (Powles and Yu 2010). Although we did not carry out specific assays to determine the effects of the *Ahasl1-1* and *Ahasl1-3* substitutions on the AHAS K_m (pyruvate) values, the results reported for different AHAS resistance mutations in other plant species support the hypothesis of differences in affinity to pyruvate (Chang and Duggleby 1998; Preston et al. 2006). This allows to explain why the same AHAS inhibition curves for homozygotes *Ahasl1-1* and heterozygotes *Ahasl-3* led to significant differences in their biomass response to increasing doses of imazapyr.

In summary, the results of this study indicate that the AHAS resistant alleles *Ahasl1-1* and *Ahasl1-3* showed significantly differences in their response to imazapyr application, either in homozygous or heterozygous states. Genotypes carrying the *Ahasl1-3* allele are more tolerant at the phenotypic (PI and biomass accumulation) and enzymatic levels than those carrying the *Ahasl1-1* allele. Dominance relationships at the *Ahasl1* locus indicated that both resistant alleles are recessive with respect to the wild-type allele at the enzymatic level but they showed from dominance to recessivity at the phenotypic level. This discrepancy can be explained by the margin of error of the enzyme and by differences in AHAS functionality of each of the tolerance-conferring AHAS gene mutations. Interestingly, *Ahasl1-3* showed dominance over *Ahasl1-1* both at the phenotypic and enzymatic levels and at all the tested doses, an observation that can be interpreted taking into account the protein structure of the AHAS catalytic subunit.

Practical implications of these results are important in two different technological areas. From a plant breeding perspective, and given the dominance relationships between *Ahasl1-1* and *Ahasl1-3* alleles, homozygous *Ahasl1-3/Ahasl1-3* genotypes will show the same level of imidazolinone tolerance than heterozygous *Ahasl1-1/Ahasl1-3* genotypes. For this reason, both types of materials can be used in the development and production of commercial hybrids with higher levels of tolerance than homozygous *Ahasl1-1* (Imisun) hybrids. Likewise, to obtain a CLPlus hybrid from an Imisun hybrid, only one of the parental lines of the later genotype should be converted by substituting the *Ahasl1-1* allele by *Ahasl1-3*. In the framework of tolerance weed management the obtained results indicate that using low herbicide rates permit heterozygotes to survive and multiply, so this practice should be avoided in order to select against newly arisen herbicide resistant alleles in weed populations. Second, dominance relationships at the *Ahasl1* locus of sunflower permits to explain how moderately resistant mutations can be maintained in natural populations of weed species.

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