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Differential expression of acetohydroxyacid synthase genes in sunflower plantlets and its response to imazapyr herbicide

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ABSTRACT

Acetohydroxyacid synthase (AHAS) catalyzes the first reaction in branch chain amino acids biosynthesis. This enzyme is the target of several herbicides, including all members of the imidazolinone family. Little is known about the expression of the three acetohydroxyacid synthase genes (*ahas1*, *ahas2* and *ahas3*) in sunflower. The aim of this work was to evaluate *ahas* gene expression and AHAS activity in different tissues of sunflower plantlets. Three genotypes differing in imidazolinone resistance were evaluated, two of which carry an herbicide resistant-endowing mutation known as *Ahasl1-1* allele. *In vivo* and *in vitro* AHAS activity and transcript levels were higher in leaves than in roots. The *ahas3* transcript was the less abundant in both tissues. No significant difference was observed between *ahas1* and *ahas2* transcript levels of the susceptible genotype but a higher *ahas1* transcript level was observed in leaves of genotypes carrying *Ahasl1-1* allele. Similar transcript levels were found for *ahas1* and *ahas2* in roots of genotypes carrying *Ahasl1-1* allele whereas higher *ahas2* abundance was found in the susceptible genotype. Herbicide treatment triggered tissue-specific, gene and genotype-dependent changes in *ahas* gene expression. AHAS activity was highly inhibited in the susceptible genotype. Differential responses were observed between *in vitro* and *in vivo* AHAS inhibition assays. These findings enhance our understanding of AHAS expression in sunflower genotypes differing for herbicide resistance and its response to herbicide treatment.

1. Introduction

Acetohydroxyacid synthase (AHAS; EC 4.1.3.18) also known as acetolactate synthase is the first enzyme in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine. This enzyme catalyzes two reactions, in which either 2-acetolactate or 2-aceto-2-hydroxybutyrate is formed [1]. The biosynthesis of the branched chain amino acids primarily occurs in plastids of young tissues of different plant organs [2,3].

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

The number of *ahas* genes varies according to the plant species. *Arabidopsis thaliana* contains one gene which is constitutively expressed [6]. Multiple *ahas* genes have been identified in several other plant species: six in cotton [7], five in oilseed rape [8], three in wheat [9], two in tobacco [10], corn [11] and several weed species [12–18]. Expression patterns of *ahas* genes have not been studied in most cases.

AHAS activity and *ahas* transcript accumulation were detected in roots and leaves from young plantlets and developing reproductive tissue [19]. A coordinated expression of the two *ahas* genes was observed in tobacco, mainly in developing organs such as inflorescences and root apices [10]. On the other hand, tissue-specific expression was found for different members of *ahas* gene family in cotton and oilseed rape [7,20]. A recent work examined transcription level of two *ahas* genes through RT-qPCR in *Echinochloa phyllopogon*. A differential contribution of each gene was observed in leaves and roots [15].

Three genes coding for the AHAS catalytic subunit have been identified in sunflower (*ahas1*, *ahas2* and *ahas3*). Expression of the three members was detected but it could not be quantified [21,22]. Moreover, AHAS activity in sunflower was studied only in leaf tissues [23–26].

Abbreviations: AHAS, acetohydroxyacid synthase; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

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A single base-pair change in the gene encoding the large subunit of AHAS, and the consequent single amino acid change in the mature protein, results in an imidazolinone-resistant enzyme [27]. Several different mutations in *ahas1* have been identified that confer imidazolinone resistance in sunflower. Specifically, the amino acid changes identified are Ala205Val (amino acid number in reference to AHAS sequence from *A. thaliana*) [22,28], Trp574Leu [29] which was discovered in wild biotypes, and Ala122Thr which was obtained through seed mutagenesis [30]. Mutation at Ala205, also known as Imr_1 [31], Ar_{pur} [22] or *Ahasl1-1* allele [30], does not confer complete resistance to imidazolinone herbicides [31]. A second modifier gene (Imr_2) is necessary to achieve complete resistance [31]. The effect of the modifier *locus*, Imr_2, remains unknown and it might be related to an altered AHAS expression or non-target-site resistance (herbicide uptake, transport or metabolism).

The objective of this work was to measure the transcript levels of the three *ahas* genes and AHAS activity in young leaf and root tissues of sunflower lines differing in herbicide resistance. In addition, the response to imidazolinone herbicide treatment was evaluated at the transcriptional and enzyme activity levels.

2. Materials and methods

2.1. Plant materials

Three sunflower inbred lines were used in this study: HA425, 1058-1 and HA89 which are imidazolinone resistant (*Ahasl1-1Ahasl1-1* Imr_2Imr_2), intermediate (*Ahasl1-1Ahasl1-1* imr_2imr_2) and susceptible (*ahasl1ahasl1* imr_2imr_2), respectively. The resistant line HA425 was developed and released by the USDA-ARS and the North Dakota Agricultural Experiment Station [32]. The line 1058-1 was developed from a backcross between HA425 and HA89 [31].

2.2. Plant growth conditions and tissue collection

Achenes were planted on plastic pots (4 cm wide, 5.5 cm high) filled with commercial perlite and watered by capillarity with nutritive solution consisting of Murashige and Skoog's [33] medium (25% v/v). Pots were incubated in growth chamber under controlled conditions ($25 \pm 2\,^{\circ}C$, 16 h light, $100\,\mu mol\,m^{-2}\,s^{-1}$). After 14 days, the seedlings were dissected into roots and leaves and immediately used for AHAS activity assays or RNA extraction. Commercial herbicide imazapyr (Clearsol®, BASF) was applied to nutritive solution to a final concentration of 10 μM 48 h before harvest.

2.3. Relative RT-qPCR

RNA was extracted from leaf and root tissues using the PureLink™ RNA Mini Kit (Invitrogen Life Technologies) according to the instructions provided by the manufacturer in two independent experiments. DNaseI treatment was performed using on-column PureLink™ DNase (Invitrogen Life Technologies). RNA was quantified using a spectrophotometer and integrity was confirmed through gel electrophoresis. RNA was stored at −80 °C until used. First strand cDNA synthesis was performed with the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. Reverse transcription was initiated using oligo-dT supplied with the kit.

Sunflower *ahas* sequences (accession numbers AY541451–AY541458) and ESTs (DY909668.1, DY906644.1, DY912682.1) were gathered from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Due to the high similarity between *ahas1* and *ahas2* it was difficult to design paralog-specific primers. Therefore, *ahas1* forward primer was designed based on the 5′ (untranslated) UTR region. Primers were designed using the Primer 3 [34] and Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer pairs corresponding to *ahas1*, *ahas2*, *ahas3*, and *β-tubulin* are listed in Table 1. Real-time PCR analysis was performed using the Rotor-Gene Q (Qiagen®) thermal cycler. The reaction contained 1x SYBR Green PCR Master Mix (Mezcla Real®, Biodynamics, Argentina), 0.4 μM of the forward and reverse primers and 1.5 μL of cDNA (1:5 dilution) in a total volume of 15 μL. No-template controls were also included. Each qPCR cycle consisted of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 40 s at 72 °C. RT-PCR products were sequenced to confirm the gene specific amplification. All reactions were run in triplicate. Quantification cycle (Cq) and efficiency (*E*) for each amplicon were obtained from the Comparative Quantitation software supplied by Corbett Research for Rotor Gene. *β-tubulin* was selected as a suitable reference gene in roots and leaves in control and herbicide treatments (Cq for tubulin showed a standard deviation lower than 1 under all conditions) [35]. This gene was already used as reference gene in RT-qPCR experiments [36] and was probed to be stable [37]. Normalized expression value for each gene was calculated based on E and Cq in comparison to the reference gene according to Simon's formula [38]. Relative expression ratios were calculated considering control samples and samples from herbicide treated plants [39].

Table 1
Oligonucleotide primers used for relative RT-qPCR.

Target gene	Forward primer 5′–3′	Reverse primer 5′–3′
ahas1	CGAACGGTAACCCTAGAACAC	AAGTGATGGGTAATGCGAAAC
ahas2	GCCGCCATACATCCTCCCC	CCGGTGGTGGTGGTGGAGTC
ahas3	GTTGTTGGCGTTTGGCGTCCG	GATCGCGTTCCCACCCGTCAC
β-tubulin	TCTGCCACCATGTCGGGAGTT	GTAACGCCCGTGTCGTGGGTC

2.4. In vitro AHAS assay

AHAS *in vitro* activity was determined following the procedure described by Yu et al. [40] and Degrande et al. [19] with modifications. Plant tissues were powdered in liquid nitrogen and suspended in $5\,mL\,g^{-1}$ fresh weight of buffer containing 50 mM N(2-hydroxyethyl)-piperazine-N9-(2-ethanesulfonic acid), pH 7, 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate, and 20 μM flavin adenine dinucleotide. Insoluble polyvinylpolypyrrolidone (PVPP) was added at the ratio of tissue:insoluble PVPP 6:1. The homogenate was centrifuged at $27{,}000 \times g$ for 15 min, and immediately used for enzyme activity assays. Crude extract (400 μL) and the same volume of distilled water were incubated at 37 °C for 60 min. Afterwards, the content of the reaction tube was divided in two aliquots of 350 μL. In one of the aliquots, the reaction was stopped by adding 70 μL of 5.5 N H_2SO_4 and incubated at 60 °C for 15 min to convert acetolactate to acetoin. Acetoin-forming enzymes in plant tissues may interfere with the assay [41], 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 [42]. Acetoin was quantified by a modified colorimetric assay [43] wherein 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 ($11{,}000 \times g$). Absorbance was measured spectrophotometrically at 530 nm. AHAS activity was calculated as the mean of three independent repetitions and expressed as absorbance at $530\,nm\,g^{-1}$ fresh weight h^{-1}.

2.5. In vivo AHAS assay

AHAS *in vivo* activity was determined by the procedure described by Gerwick et al. [44] and Simpson et al. [45] with modifications. A stock incubation solution containing 25% (v/v) of Murashige and Skoog's [33] medium, 0,025% Triton X-100, 500 μM 1,1-cyclopropanedicarboxylic acid (CPCA) and 0 (control) and 10 μM imazapyr was prepared immediately before each assay. Petri dishes containing detached leaves (200 mg) 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. It was not possible to detect activity in detached roots. Therefore, for root activity determination, the incubation solution was applied as watering solution in intact plants for 24 h. After incubation, leaves and roots were weighted and stored at −20 °C until used. Tissue was grounded in liquid nitrogen with 1 mL of water per gram of fresh weight and 100 mg of PVPP. The samples were incubated at 60 °C for 15 min followed by incubation at room temperature for 45 min, and vortexing every 15 min to facilitate enzyme products extraction from leaf and root tissues. After centrifugation at 4 °C for 10 min (11,000 × *g*) a 400-μL aliquot was taken and mixed with 40 μL of 5.5 N H_2SO_4. The mixture was incubated at 60 °C for 30 min to facilitate descarboxilation of enzyme products. The contribution of the direct formation of acetoin by non-AHAS activities was determined using NaOH instead of H_2SO_4. Acetoin and 3-hydroxy-2-pentanone were quantified by a modified colorimetric assay previously described [43]. Absorbance of the 2,3-diketone species was measured spectrophotometrically at 530 nm [46]. AHAS activity was calculated as the mean of three independent repetitions and expressed as absorbance at 530 nm g^{-1} fresh weight h^{-1}.

2.6. Data analysis

Data were tested for statistical significance using analysis of variance. Normality of the empirical distribution of each variable was assessed by the Shapiro–Wilk (*W*) test. Homogeneity of variance was evaluated using the Levene's test on absolute residuals. Logarithmic transformations were used to stabilize the variances. Means from different tissues and genotypes were compared using Tukey's multiple comparison test. Control and herbicide treatment means were compared using Student's *t*-test. Statistical analyses were performed using *agricolae* and *car* packages of R software [47].

3. Results

3.1. Transcriptional levels of ahas genes

The expression pattern of sunflower *ahas* gene family is showed in Fig. 1. The *ahas3* transcript was the less abundant in both tissues. For *ahas1* and *ahas3*, the transcript levels were ten times higher in leaves than in roots. In root tissues, the transcript level of *ahas1* was not significantly higher than other gene paralogs. A higher *ahas1* transcript level was observed in leaves of intermediate and resistant genotypes. Only for the susceptible line, no significant difference was found between *ahas1* and *ahas2* transcript abundance in leaves. Moreover, *ahas2* showed the highest transcript level in roots of this genotype.

Herbicide treatment triggered significant changes at the transcriptional level of *ahas* genes except for the resistant genotype (Fig. 2). Relative transcript abundance showed less than a two-fold increase for *ahas2* in the intermediate genotype. The most noticeable change was observed for *ahas3* in roots of the susceptible and intermediate genotype, for which the transcript levels were four or five times higher in presence of imazapyr herbicide than control treatment. On the other hand, *ahas1* and *ahas3* transcription was inhibited in susceptible leaves.

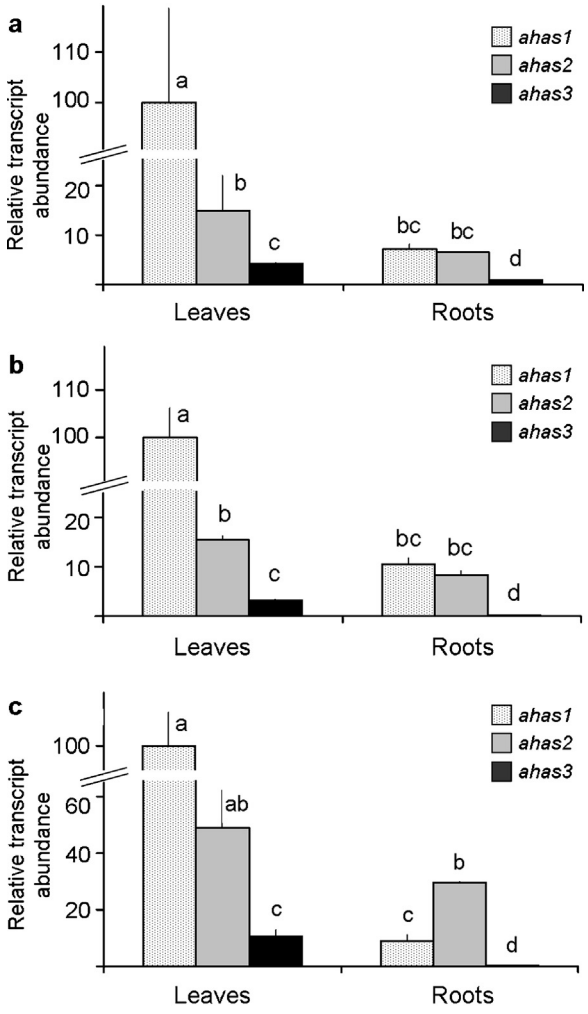

Fig. 1. Transcriptional levels of *ahas* genes (*ahas1*, *ahas2* and *ahas3*) in sunflower plantlets. Data were obtained by RT-qPCR normalized against *β-tubulin* and expressed relative to *ahas1* in leaves for the three evaluated genotypes HA425 (a), 1058-1 (b) and HA89 (c). Vertical bars indicate SE of the mean. Mean values with the same letter are not significantly different at the 0.05 probability level (Tukey's multiple comparison test). Data were log-transformed; letters refer to transformed mean values.

3.2. AHAS activity

AHAS activity in sunflower plantlets was measured through *in vivo* and *in vitro* assays (Fig. 3). Similar patterns were observed for the three evaluated genotypes. Higher levels of activity were detected in leaf than in root tissues.

In vitro AHAS activity in herbicide treated plantlets decreased significantly compared to control only for leaves of the susceptible line HA89 ($p < 0.05$) (Fig. 4). Activity in leaves of this genotype was less than 5%. By contrast, the extractable activity in root tissues showed a reduction of about 40% (Fig. 4).

There was a significant decrease of *in vivo* AHAS activity ($p < 0.05$) in both leaf and root tissues. The susceptible line showed the lowest values of AHAS activity. The intermediate and resistant genotypes showed a similar response (Fig. 4).

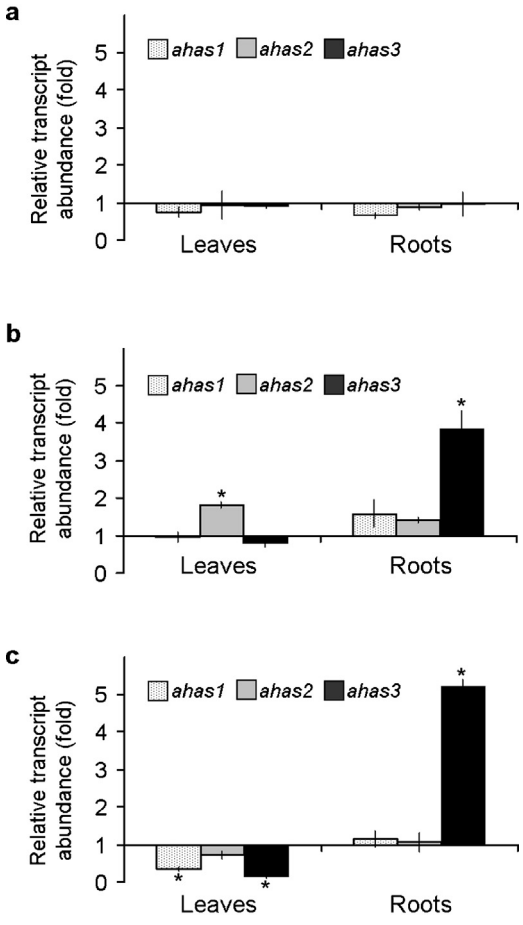

Fig. 2. Transcriptional levels of *ahas* genes (*ahas1*, *ahas2* and *ahas3*) in sunflower plantlets treated with imazapyr herbicide. Data from RT-qPCR were normalized against *β-tubulin* and expressed relative to control conditions for the three evaluated genotypes HA425 (a), 1058-1 (b) and HA89 (c). Vertical bars indicate SE of the mean. *Herbicide treatment differs significantly from control by *t*-test ($p < 0.05$) on the log-transformed values.

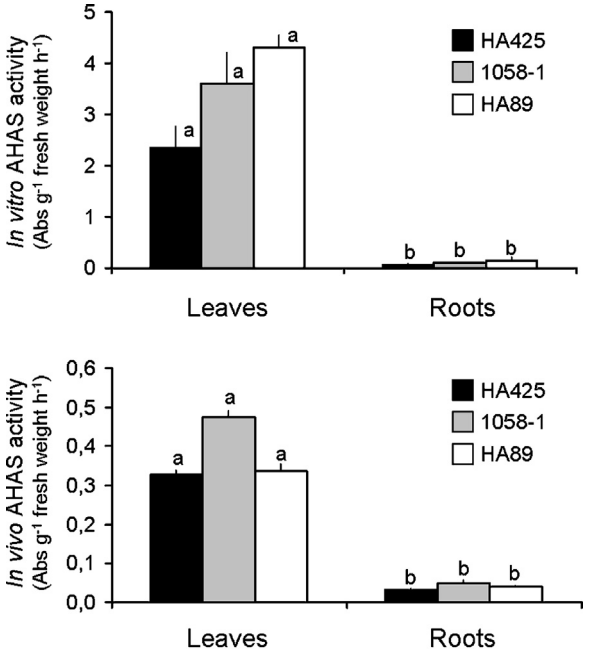

Fig. 3. Acetohydroxyacid synthase (AHAS) activity in sunflower plantlets for the resistant (HA425), intermediate (1058-1) and susceptible (HA89) genotypes. Activity was expressed as absorbance at 530 nm (g fresh tissue)$^{-1}$ h^{-1} for both *in vitro* and *in vivo* assays. Vertical bars indicate SE of the mean. Mean values with the same letter are not significantly different at the 0.05 probability level (Tukey's multiple comparison test). Data were log-transformed; letters refer to transformed mean values.

4. Discussion

4.1. Transcriptional levels of ahas genes

In this work, *ahas* transcript levels were measured using relative RT-qPCR in sunflower plantlets of three inbred lines differing in resistance to imidazolinone herbicides. Previous works could not accurately quantify differences in expression among paralogs because of several technical problems due to the high conservation of this gene family [21,22].

The obtained results are consistent with a previous non-quantitative RT-PCR study, which found that *ahas1* and *ahas2* were expressed in several tissues whereas *ahas3* was more weakly expressed than the other two genes [21].

The relative transcript level did not significantly differ between *ahas1* and *ahas2* in leaves of the susceptible line. Moreover *ahas2* showed a higher abundance than *ahas1* in roots of susceptible line. The resistant and intermediate genotypes showed a higher transcript level of *ahas1* (Fig. 1). These genotypes carry the *Ahasl1-1* allele (Ala205 mutation). Ala205 mutation was found to reduce the specific AHAS activity in other species [48]. Therefore, a higher transcriptional level may compensate the reduced functionality of this isoform. Similarly, mutant barley *ahas* showed a higher expression level that contributes to the full AHAS activity of the plant [49].

At present, the induced and natural mutations conferring imidazolinone resistance in sunflowers were identified in *ahas1* [21,22,28–30]. For that reason, it was suggested that *ahas1* gene was the gene family member that encodes the AHAS enzyme with essential housekeeping functions in sunflower and that it is predominantly expressed in tissues affected by herbicide treatment [21,22,30]. However, from the results found in the present work,

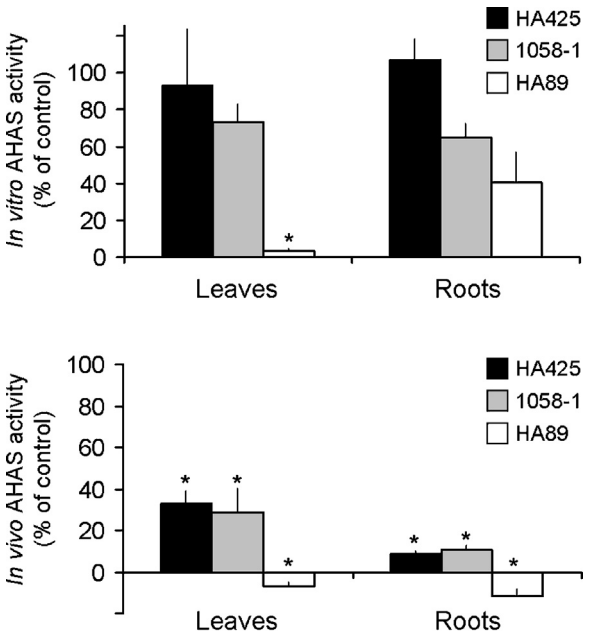

Fig. 4. Acetohydroxyacid synthase (AHAS) activity in sunflower plantlets treated with imazapyr herbicide for the resistant (HA425), intermediate (1058-1) and susceptible (HA89) genotypes. Activity was expressed as percentage of the control for both *in vitro* and *in vivo* assays. Vertical bars indicate SE of the mean. *Herbicide treatment differs significantly from control by *t*-test ($p < 0.05$) on the log-transformed values.

it becomes evident the importance of isoforms other than AHAS1. Further analysis, including additional tissues and developmental stages, should be done for a better understanding of the expression pattern of the *ahas* gene family in sunflower. In particular, experiments are being conducted to assess transcript levels in reproductive tissues given that specific AHAS isoforms were found in cotton and oilseed rape [7,20]. Furthermore, sterility observed in resistant sunflowers treated with imidazolinone at reproductive stages suggests that isoforms different from AHAS1 were inhibited by herbicide action in reproductive tissues [50].

Herbicide response was not uniform for the three genotypes evaluated here. The effective herbicide action was probably different in each genotype, as they differ in herbicide resistance level. Therefore, each genotype showed a distinct response in *ahas* transcript abundance. In *Arabidopsis*, a resistant genotype treated with imazapyr did not exhibit significant changes in the transcriptome while the wild type showed numerous changes including a slight increase in the expression of *ahas* [51]. A tissue-specific and gene-dependant response was also observed in the present study. Transcription of each *ahas* gene in sunflower seems to be subjected to a different regulatory mechanism. *Ahas3* showed the higher induction in relative transcript abundance after imazapyr treatment in roots of susceptible and intermediate genotype. *Ahas2* displayed a small degree of induction only in leaves of the intermediate genotype. In addition, a significant reduction in *ahas1* and *ahas3* transcript levels occurred for leaves of susceptible genotype (Fig. 2). Similarly, an inhibitory effect on the *ahas* transcript levels was observed in leaves but not in roots of rice plantlets treated with imazethapyr [52].

4.2. AHAS activity

AHAS activity could be measured through both *in vitro* and *in vivo* assays. This is the first report of an *in vivo* assay for roots. *In vivo* techniques are cost-effective and simple tools for a rapid measure of total AHAS activity and also might be employed as a diagnostic test. As opposed to the *in vitro* techniques performed on the isolated enzyme in a test tube, the *in vivo* assay uses an inhibitor of the ketoacid reductoisomerase (KARI), the enzyme that acts after AHAS in the branched-chain amino acid biosynthetic pathway. Inhibition of KARI results in an accumulation of AHAS's reaction products. AHAS activity is indirectly measured by converting AHAS products to chromophores that are then quantified colorimetrically [44].

For both, *in vivo* and *in vitro* activity, similar results were obtained and also were consistent with *ahas* transcriptional levels (Fig. 1,3). In all cases, AHAS expression was higher in leaves than in roots. This pattern was also found for other species such as corn, wheat, sorghum, and barley [53–56] whereas in oilseed rape and chicory comparable AHAS activity was observed in both tissues [19,56]. Nevertheless, differences between plant tissues changed at various growth stages. In 14-day-old lima bean plants, the highest levels of AHAS activity were found in leaves but roots showed a higher activity than leaves with increasing plant age [57].

AHAS activity inhibition (Fig. 4) was similar to those reported for wild sunflower in the *in vitro* [23,24] and *in vivo* [26] technique. Inhibition by herbicide action was more pronounced in the *in vivo* than *in vitro* assay (Fig. 4). Lower herbicide resistance levels in the *in vivo* assays compared to *in vitro* responses were also described in other species and could be explained by environmental effects or other factors besides target site insensitivity, such as herbicide uptake, transport, or metabolism mediated plant responses to AHAS inhibitors [58,59].

The levels of *in vitro* AHAS activity in roots of the susceptible genotype were less affected by the herbicide even though herbicide was applied in the nutrient solution (Fig. 4). A similar behavior was observed in corn, wheat and lima bean [57,60,61]. The differential response between leaf and root tissues could be related to the response at transcriptional level, for which *ahas* genes transcription was inhibited in leaves but induced in roots (Fig. 3). A larger amount of enzyme in roots could dilute the effect of herbicide molecules, giving a reduced inhibition.

The susceptible genotype is recessive homozygous for *ahas1* (*ahasl1ahasl1*) while intermediate and resistant genotypes have the same constitution at *ahas1 locus* carrying resistant alleles (*Ahasl1-1Ahasl1-1*). These differences were reflected in AHAS activity, since the resistant and intermediate genotypes showed the same pattern of activity while the susceptible line was more affected by herbicide action (Fig. 4). Besides, *in vivo* activity was more inhibited in roots than in leaves for the intermediate and resistant genotypes. This result could be explained by the presence of susceptible isoforms encoded by *ahas2* in the enzyme pool, as similar transcript levels were found for *ahas1* and *ahas2* in roots. In the same way, these results are consistent with previous findings at phenotypic level since root growth parameters were more sensitive when screening for herbicide resistance [62,63].

Intermediate (*Ahasl1-1Ahasl1-1 imr$_2$imr$_2$*) and resistant (*Ahasl1-1Ahasl1-1 Imr$_2$Imr$_2$*) genotypes differ at *Imr$_2$ locus*. Given that no differences in AHAS expression were found between intermediate and resistant genotypes, the possibility that *Imr$_2$* could be associated with higher levels of AHAS expression is excluded.

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