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Root biomass response to foliar application of imazapyr for two imidazolinone tolerant alleles of sunflower (*Helianthus annuus* L.)

Carlos A. Sala*, Mariano Bulos, Emiliano Altieri and María Laura Ramos

Department of Biotechnology, Nidera S.A., Casilla de Correo 6, CP.: 2600 Venado Tuerto, Santa Fe, Argentina

Imisun and CLPlus are two imidazolinone tolerance traits in sunflower (*Helianthus annuus* L.) determined by the expression of two alleles at the locus *Ahasl1*. Both traits differed in their tolerance level to imazapyr —a type of imidazolinone herbicide— when aboveground biomass is considered, but the concomitant herbicide effect over the root system has not been reported. The objective of this work was to quantify the root biomass response to increased doses of imazapyr in susceptible (*ahasl1/ahasl1*), Imisun (*Ahasl1-1/Ahasl1-1*) and CLPlus (*Ahasl1-3/Ahasl1-3*) homozygous sunflower genotypes. These materials were sprayed at the V2–V4 stage with increased doses of imazapyr (from 0 to 480 g active ingredient ha^{-1}) and 14 days after treatment root biomass of each plant was assessed. Genotype at the *Ahasl1* locus, dose of imazapyr and their interaction significantly contributed ($P < 0.001$) to explain the reduction in root biomass accumulation after herbicide application. Estimated dose of imazapyr required to reduce root biomass accumulation by fifty percent (GR_{50}) differed statistically for the three genotypes under study ($P < 0.001$). CLPlus genotypes showed the highest values of GR_{50}, 300 times higher on average than the susceptible genotypes, and almost 8 times higher than Imisun materials, demonstrating that both alleles differ in their root biomass response to foliar application of increased doses of imazapyr.

Key Words: root growth, herbicide tolerance, *AHAS*, breeding, imidazolinones.

Introduction

Sunflower (*Helianthus annuus* L. var. *macrocarpus* Ckll.) is grown all over the world with three main purposes: beauty (ornamental sunflower), direct consumption of the seeds (confectionary sunflower) and oil production (oilseed sunflower). By far, the last of them is the most important objective in terms of acreage and production (Miller and Fick 1997). Sunflower oil has been traditionally viewed as a healthful vegetable oil and it is considered premium oil for salad, cooking and margarine production (Dorrell and Vick 1997) and also is being evaluated as a source of biodiesel (Bunta and Mario 2008). Weeds compete with sunflower for moisture, nutrients, and depending on species for light and space. Weed competition cause substantial yield losses in sunflower, with reports ranging from 20 to 70% (Bedmar *et al.* 1983, Brighenti *et al.* 2004, Chubb 1975, Fleck *et al.* 1989, Robinson 1973). Herbicides are the most desirable method for weed control, however the availability of selective herbicides for the sunflower crop is quite limited and, due to the high cost of herbicide registration, new molecules of herbicides are unlikely to be specifically developed for weed control in sunflower. For this reason, gene discovery and trait development for herbicide resistance in this crop, particularly imidazolinones and sulfonylureas, was an active area of research during the past decade (Sala *et al.* 2012b).

Imidazolinone and sulfonylurea herbicides have been demonstrated to have a broad spectrum of weed control activity, flexibility in timing of application, low usage rates, and low mammalian toxicity (Brown 1990, Tan *et al.* 2005). These herbicides inhibit the enzymatic activity of acetohydroxyacid synthase (AHAS, EC 4.1.3.18 also known as acetolactate synthase, ALS; Ray 1984, Shaner *et al.* 1984), the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine and isoleucine (Singh 1999).

The first commercial imidazolinone tolerance trait in sunflowers is known as 'Imisun' and it was developed from an imidazolinone-tolerant wild sunflower population crossed with cultivated sunflower (Al-Khatib *et al.* 1998). 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 (Bruniard and Miller 2001, Miller and Al-Khatib 2002). 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 an imidazolinone herbicide: imazapyr (Sala *et al.* 2008a). To achieve commercial

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*Corresponding author (e-mail: csala@nidera.com.ar)

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). Sequencing results demonstrated that *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) and *Ahasl1-3* has a G-to-A mutation in codon 122 (Sala *et al.* 2008b).

Crop injury in herbicide tolerant (HT) crops consists of several symptoms such as chlorosis, stunting, yellowing, decreased biomass production and yield loss. The crop injury phenotype can be attributed to the interaction between genotype and environment (GxE). The environmental component for herbicide tolerance is a sum of abiotic and biotic factors coupled with the effect of the type of herbicide and application parameters such as herbicide rates, surfactants and application timing (Frihauf *et al.* 2005, Geier *et al.* 2004, Stougaard *et al.* 2004). The genotypic factor in a HT plant is the sum of the HT gene(s) effect plus the remaining genetic background, and the interaction between the two. For these reasons, comparison of HT gene performances can be carry out by means of dose response experiments under environmental controlled conditions and using the same genetic backgrounds. Comparisons of different HT genes or the additive effects of HT genes controlling resistance to a given herbicide are scarce in the literature. Using the described approach, Hanson *et al.* (2006) were able to conclude that biomass accumulation after imazamox treatment was similar among tolerant winter wheat cultivars carrying the resistance genes *Ahasl1B* or *Ahasl1D*. Tolerance in this type of cultivars was always higher than that shown by spring wheat cultivars carrying the same resistant genes. It was also observed that the spring wheat cultivars carrying two resistant genes had an additive level of tolerance to imazamox compared with single-gene resistant spring wheat (Hanson *et al.* 2006). Recently, the results of a quantitative imazapyr response assay in Imisun and CLPlus homozygous sunflower lines and hybrids were reported using the same approach. A122T substitution in the *Ahasl1* gene displayed the lowest level of inhibition of the AHAS enzyme extracts by imidazolinones, which resulted in the highest level of accumulation of above-ground biomass at all rates of herbicide application. A205V substitution, on the other hand, showed a higher inhibition of AHAS activity and a moderate level of above-ground biomass accumulation (Sala *et al.* 2012a).

Nevertheless, root biomass response to increased levels of foliar imidazolinone application was not reported yet. This is surprisingly since it has been shown, for example in susceptible plants of *Arabidopsis thaliana*, that one of the earliest responses to imidazolinone treatment is the inhibition of root growth, which occurs several hours after herbicide application (Manabe *et al.* 2007). In sunflower, assessment of root growth during germination demonstrated that the susceptible genotypes showed arrested root growth at all herbicide treatments and the Imisun tolerant genotype developed a complete root system even when exposed to the highest dose of herbicide (Vega *et al.* 2009). To the best of our knowledge the impact of foliar herbicide application over root growth for different HT genes has not been reported. For this reason, the objective of this work was to quantify the root biomass response to increased doses of imazapyr in susceptible, Imisun and CLPlus homozygous sunflower genotypes.

Materials and Methods

Plant material

Three different genotypes for the *Ahasl1* locus were assessed in three different genetic backgrounds: a commercial restorer line (R20), a maintainer inbred line (BTK47) and the F_1 hybrid cmsBTK47/R20, which totalize nine genetic materials (Table 1). Susceptible genotypes (*ahasl1/ahasl1*) included the original lines BTK47, R20 and their F_1 hybrid (cmsBTK47/R20). CLPlus tolerant genotypes (*Ahasl1-3/Ahasl1-3*) included GM40, R720 and their F_1 hybrid (H3 = cmsGM40/R720). 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 (*Ahasl1-1/Ahasl1-1*) included IB9, IR7 and their F_1 hybrid H2 (= cmsIB9/IR7). IB9 traces back to BTK47 and IR7 to R20.

Table 1. Reproductive group, pedigree information, genotype for the *Ahasl1* locus, tolerance to imidazolinone and name of the trait for the utilized lines and hybrids

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

Dose response experiments

Seeds of each genotype were sown in Petri dishes and, 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 (0, 40, 80, 160, 240, 320, 400, 480 grams of active ingredient per hectare —g a.i. ha^{-1}— which corresponded to 0x, 0.5x, 1x, 2x, 3x, 4x, 5x and 6x field rates, respectively). The experiment was arranged as a randomized block design with a full factorial (sunflower line x treatment) arrangement of treatments in 10 replications.

Plants were maintained for 14 days after imazapyr treatment at which time the root biomass were recorded. To do this, each plant was extracted from its pot and the substrate was carefully washed out from the roots. Roots were dried at 60°C for 48 h for root dry weight determination. Dry biomass data were converted to percentages of the untreated control plants within each line to allow direct comparisons between groups and subjected to ANOVA using the mixed model procedure of SAS (Littell *et al.* 1996, SAS Institute 2004), with degrees of freedom calculated by Satterthwaite's approximation method (Satterthwaite 1946). Genotype at the *Ahasl1* locus was considered fixed in the model, while genetic background and imazapyr doses were considered random variables. Means were separated using Fisher's protected least significant difference (LSD) test at the 1% and 5% level of probability.

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:

$$y = 100/[1 + (x/GR_{50})^b]$$

Where y = root biomass (expressed as the percent of the untreated control), x = imazapyr dose (g a.i. ha^{-1}), 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 root biomass accumulation. Regressions were performed on all data using nonlinear least square regression procedure (PROC NLIN, SAS Institute 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 three 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.

Results

Genotype at the *Ahasl1* locus, imazapyr doses, their first order interaction and the three-factor interaction with the genetic background significantly contributed ($P < 0.001$) to the variation in root biomass accumulation 14 DAT. On the other hand, analysis of variance indicated no significant ($P < 0.05$) effect of genetic background and its two-factor interaction with genotype at the *Ahasl1* locus and imazapyr doses. Observed significant interactions suggest that differences among HT genes for their response to increased doses of imazapyr.

Plants of the susceptible inbred lines and hybrids died at any application rate of imazapyr tested showing a complete burning of the shoot apex and necrosis of the root system. Root biomass in these genetic materials decreased from 18.8 to 6.4% of the untreated control plants as the imazapyr rate increased from 40 to 480 g a.i. ha^{-1} (Table 2). The genetic materials carrying the *Ahasl1-1* allele in homozygous state showed different levels of yellowing, stunting, leaf abnormalities and necrosis according to the applied dose of herbicide. In correspondence to these phytotoxicity symptoms, root biomass significantly decreased from 59.4 to 16% of the untreated control plants when plants were challenged with increased doses of imazapyr (Table 2). In contrast, homozygous genotypes for the *Ahasl1-3* allele did not show any injury in the aboveground organs, but their root biomass also decreased from 84.6 to 51.0% of the untreated controls when they were challenged with increased doses of imazapyr, from 40 to 480 g a.i. ha^{-1}. Both tolerant genotypes, Imisun and CLPlus, significantly differed in their root biomass responses to imazapyr and these differences were expressed from the lowest to the highest doses (Table 2 and Fig. 1).

The log-logistic model accurately described root biomass response after imazapyr application for susceptible and

Table 2. Average root biomass accumulation (percentage over untreated control plants) 14 days after treatment with imazapyr on three sunflower lines or hybrids for each of three genotypes at the *Ahasl1* locus of sunflower

Doses	*Ahasl1* genotypes			
	Susceptible	Imisun	CLPlus	LSD among *Ahasl1* genotypes
	ahasl1/ahasl1	*Ahasl1-1/ Ahasl1-1*	*Ahasl1-3/ Ahasl1-3*	
0	100 a*	100 a	100 a	
40	18.8 ± 9.2 b	59.4 ± 12.2 b	84.6 ± 3.4 b	17.7
80	11.2 ± 4.0 c	50.2 ± 8.9 b	75.7 ± 7.3 c	13.0
160	10.1 ± 3.1 c	40.9 ± 5.6 c	73.0 ± 8.5 c	14.2
240	8.7 ± 1.9 c	36.0 ± 5.9 c	63.9 ± 5.5 d	11.1
320	6.6 ± 0.3 c	22.6 ± 3.5 d	61.8 ± 5.4 de	8.1
400	6.5 ± 0.3 c	18.8 ± 3.7 d	56.9 ± 5.6 e	7.4
480	6.4 ± 0.3 c	16.0 ± 4.6 d	51.0 ± 1.2 f	5.6
LSD among doses	5.6	9.2	5.2	

* mean values with the same letter do not differ among doses.

Table 3. Estimates of the doses of imazapyr needed to reduce the root biomass accumulation by the half (GR_{50}) and tolerant (T)/ susceptible (S) ratio estimated by nonlinear regression for root biomass accumulation of three genotypes for the *Ahasl1* locus in response to increasing doses of imazapyr

Type of material	GR_{50} (g.a.i ha^{-1})	GR_{50} ratio (T/S)
CLPlus	603.0 ± 59.0 a*	314.1
Imisun	78.6 ± 9.6 b	40.9
Susceptible	1.9 ± 0.7 c	1
LSD	115.3	

* mean values with the same letter do not differ among genotypes.

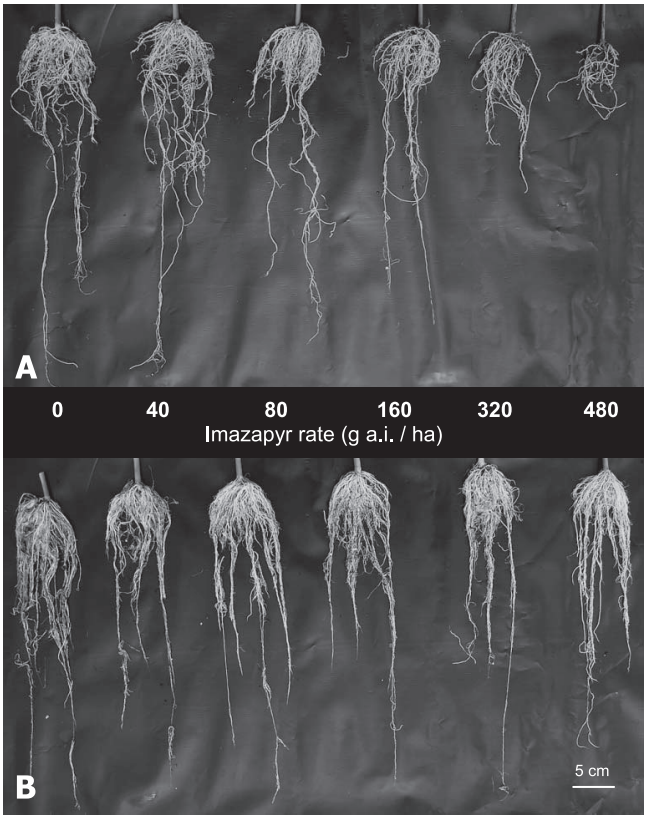

Fig. 1. Root system of sunflower plants 14 days after the application of increased doses of imazapyr. A: Imisun (*Ahasl1-1/Ahasl1-1*) plants of the inbred line IB9. B: CLPlus (*Ahasl1-3/Ahasl1-3*) plants of the inbred line GM40.

tolerant sunflower materials (Fig. 2). Estimates of the doses of imazapyr needed to reduce the root biomass of each type of genetic material by the half (GR_{50}) varied from 1.92 ± 0.75 to 603 ± 59.0 g a.i. ha^{-1}, and were statistically different among the three genotypes evaluated (Table 3). Root biomass accumulation of the susceptible materials was reduced to 50% by a dose of 1.92 g a.i. ha^{-1} of imazapyr, which represent only 2.4% of the 80 g a.i. ha^{-1} recommended as the 1x rate under field conditions. In contrast, CLPlus homozygous genotypes showed the highest values of GR_{50}, more than 300 times greater than the susceptible genotypes and 7.7 times greater than the homozygous Imisun materials

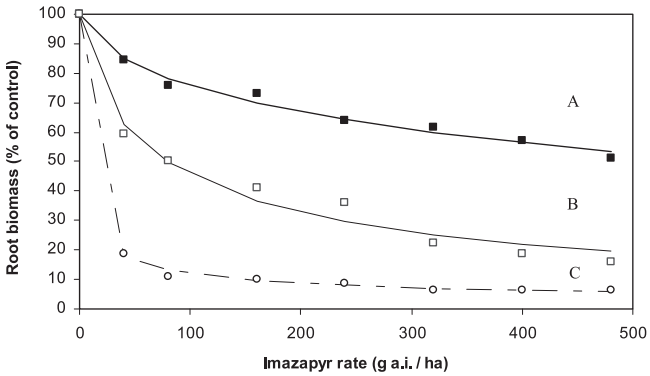

Fig. 2. Root biomass accumulation (as percentage of untreated control plants) 14 days after the application of different doses of imazapyr on three sunflower lines or hybrids for each of three genotypes at the *Ahasl1* locus of sunflower. A: CLPlus (*Ahasl1-3*/*Ahasl1-3*), B: Imisun (*Ahasl1-1*/*Ahasl1-1*), C: Susceptible (*ahasl1*/*ahasl1*)

(Table 3). On the other hand, GR_{50} estimate for the Imisun genotypes was 78.6 ± 9.6 g a.i. ha^{-1}, a dose which corresponds to a 1x application rate under field conditions.

Discussion

It was shown in many species that the biosynthesis of the branched chain amino acids primarily occurs in young tissues, a fact that is sustained by the ubiquitous accumulation of AHAS mRNAs in fast growing organs (Degrande *et al.* 2000, Singh and Matthews 1994). For example, the organs of young chicory plants (*Cichoryum intybus*) that displayed the highest AHAS activity and AHAS mRNA content are the roots and the youngest leaf. Both roots and young leaves are known to have important sink strength toward carbohydrate produced in the old leaves. Young tissues of the root and the youngest leaf are not autotrophous toward carbohydrate and mostly depend on photosynthate influx provided by older leaves (Turgeon 1989).

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. 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 (wildtype) and imidazolinone-resistant genotypes of *Arabidopsis thaliana* has demonstrated that in wildtype 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 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).

The results obtained in this study showed that root growth of susceptible plants of sunflower is inhibited by low doses of imazapyr. In fact, a dose of imazapyr of 1.92 g a.i. ha^{-1} reduced by 50% the biomass of the roots 14 DAT, a value that is almost the same of that reported for the inhibition of the aboveground organs with the same herbicide ($GR_{50} = 1.9$; Sala *et al.* 2012a). This indicates that the inhibition of AHAS activity in susceptible plants should be similar in both roots and young leaves.

Interestingly, the genetic background effect and its first order interactions with herbicide doses and genotype at the *Ahasl1* locus, did not contribute significantly to the observed variability in root biomass responses; an observation also reported for the aboveground biomass accumulation after herbicide treatment in wheat (Willenborg *et al.* 2008) and sunflower (Sala *et al.* 2012a). However, the alleles that confer tolerance to imidazolinones in sunflower, *Ahasl1-1* and *Ahasl1-3*, showed significantly different root biomass responses to increased doses of imazapyr. In fact, the estimated value for GR_{50} was almost 8 times higher for plants carrying the *Ahasl1-3* allele than those carrying the *Ahasl1-1* allele. The accumulation of root biomass after two weeks of herbicide application for both genotypes was highly associated with the already reported *in vitro* AHAS inhibition kinetics with imazapyr (Sala *et al.* 2012a). In fact, plants carrying the *Ahasl1-3* allele in homozygous condition displayed the lowest level of inhibition of the AHAS enzyme extracts which would result in the higher level of accumulation of root biomass at all rates of herbicide application. Plants homozygous for the *Ahasl1-1* allele, on the other hand, showed a higher inhibition of AHAS activity and hence, a moderate level of root biomass accumulation after IMI application.

Interestingly, GR_{50} estimated value for the root biomass response to imazapyr in CLPlus genotypes (603 ± 59) was almost the same as the corresponding GR_{50} value for shoot biomass response already reported (658.4, Sala *et al.* 2012a) indicating that this genotype shows the same pattern of response to increased levels of imidazolinones for shoots and roots. However, for the Imisun genotypes the pattern of response for the shoot is three times higher than for the root ($GR_{50} = 78.57$ g a.i. ha^{-1} for root biomass and 233 g a.i. ha^{-1} for shoot biomass) indicating that biomass accumulation in the roots for this genotype is more sensitive to imidazolinone application than biomass accumulation in the shoots. Since Imisun genotypes need an enhancer factor to achieve high levels of tolerance appart from the target tolerance conferred by *Ahasl1-1* (Bruniard and Miller 2001), it is tempting to speculate that this factor is expressed basically in the shoots, but this issue deserves to be fully investigated previous to reach to any conclusion. Nevertheless, this result indicates that the level of biomass accumulation after herbicide application in the aboveground parts of a HT plant may not be associated with the corresponding level of biomass accumulation in the roots and that both variables should be empirically assessed when comparing two HT traits of a given crop.

The results obtained clearly show that CLPlus genotypes are more tolerant to imidazolinones than Imisun genotypes at the root level when evaluated under non-stress conditions. In *Arabidopsis*, it has been shown that non target genes involved in the response to imidazolinone in wild type plants (for example, glutathione transferase (GST), cytochrome P450, ATP-binding cassette (ABC) transporter, multidrug and toxin extrusion (MATE) and alternative oxidase (AOX) protein families) also function in other abiotic stress responses (Manabe *et al.* 2007). This raises the possibility that abiotic stress and imidazolinone treatment may have additive effects that result in plant death or severe injury at lower concentrations of imidazolinone application. It is likely that the combined effect of imidazolinone application and environmental stresses under field conditions might result in even greater differences between CLPlus and Imisun sunflowers.

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