Imisun tolerance is the result of the interaction between target and non-target tolerance mechanisms

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ABSTRACT

- Imidazolinone tolerance in Imisun sunflowers has been reported to be genetically controlled by two genes, *Imr*$_1$ and *Imr*$_2$. It was shown that *Imr1* is an *Ahasl1* mutant which harbors an A205V substitution conferring a target type tolerance. The nature of the tolerance endowed by *Imr*$_2$ has not been determined yet.
- Plants of two Imisun lines, IMI-1 and RHA426, were randomly assigned to two pre-treatments, one consisting in the application of 1 ml/L of malathion ―an inhibitor of P450 activity― and the other, used as control, consisting in the application of the same volume of water. Four hours after these initial pre-treatments, plants were distributed into six treatments consisting in the application of increased doses of imazapyr, from 0 to 640 g a.i. ha^{-1}. Plants of two Clearfield Plus (genotype *Ahasl1-3/Ahasl1-3*) and one susceptible (*ahasl1/ahasl1*) lines received exactly the same treatments and were used as controls. The experiment was replicated twice. Fourteen days after treatments (DAT), dry matter content of the above-ground biomass of each plant was assessed. Dose response curves were fitted for each genotype and replication, and the estimated value of the dose of herbicide needed to reduce the dry matter weight by the half (GR_{50}) was obtained.
- Susceptible plants died 7-10 DAT after the application of any of the herbicide doses. Both Clearfield Plus genotypes showed exactly the same response to increased levels of imazapyr application, with GR_{50} values greater than 1000. No significant differences in GR_{50} values could be detected after the malathion pre-treatment in these genotypes. Imisun lines, on the other hand, showed striking differences in their response to imazapyr. As a matter of fact, GR_{50} estimation for RHA426 was twice times greater than the GR_{50} value estimated for the inbred line IMI-1. A significant reduction of 15% was observed for the GR_{50} value of IMI-1 after malathion treatment. This effect was not detected for RHA426.
- Consistently with previous results, Clearfield Plus lines showed a strong level of tolerance to increased doses of imazapyr. This high tolerance level was not modified by the inhibition of P450s by malathion. Both Imisun lines have the same genotype at the *Ahasl1* locus (*Ahasl1-1/Ahasl1-1*) and the same haplotype for the *Ahasl2* and *Ahasl3* loci. Differences in their response to increased levels of imazapyr can only be explained by the presence of non-target mechanisms interacting with the AHAS endowed tolerance. Moreover, these differences indicate that this non-target mechanism account for almost 50% of the GR_{50} values of both lines. One of the putative mechanisms of non target tolerance ―the inhibition of P450 activity― only explained 15.7% of the GR_{50} value in one of the Imisun lines. These suggest that other mechanisms in addition to the P450 activity are of greater importance in controlling the tolerance in Imisun sunflowers. Hence, the genetic control of this trait seems to be more complex than already postulated. However, it can be concluded that the previously described gene *Imr*$_2$ (*E* factor, or enhancer) can control P450 activity in certain cases but that other non-target mechanisms should also be present to achieve adequate levels of tolerance in Imisun sunflowers.
- Imisun sunflowers represent the first example of a commercial herbicide tolerance trait conferred by two independent tolerance mechanisms. Perhaps the intermediate level of tolerance endowed by the target type tolerance (A205V substitution) permitted to identify the second system. Nevertheless, the physiological mechanisms involved in the tolerance of Imisun sunflowers permit to explain the phytotoxicity variability observed among sunflower hybrids carrying the *Ahasl1-1* allele in homozygous state when challenged with imidazolinones. On the other hand, the rather complex nature of the non-target tolerance mechanism involved in Imisun tolerance permits also to explain the difficulties arising when converting conventional lines to their tolerant counterparts using solely a molecular marker based approach.

Key words: AHAS, breeding for tolerance, herbicide tolerance, imidazolinone, P450.

INTRODUCTION

There are two primary mechanisms of herbicide tolerance in plants: (i) tolerance caused by mutations in target sites of the herbicide (*target-site tolerance*), and (ii) tolerance caused by mutations in non-target sites (*non-target site tolerance*). Target-site tolerance involves a reduced sensitivity of target specific enzymes or proteins and, thus, this type of tolerance is mostly monogenic (Devine & Eberlein, 1997). Non-target tolerance, on the other hand, involves several mechanisms, such as reduced uptake or translocation of the herbicide, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site into the vacuole or the apoplast (Owen and Zelaya, 2005; Tranel and Wright, 2002; Yuan et al., 2007). From a biochemical perspective, non-target herbicide tolerance can be caused by detoxification processes which involve the oxidation of the herbicide molecules, typically carried out by P450 monooxygenases or mixed function oxidases, conjugation of the activated xenobiotic using thiols or sugars, transporting the conjugated molecule into the vacuole or extracellular space by active transport by means of ABC transporters, and degradation of the conjugated molecule into the vacuole or extracellular spaces. Many plant detoxifying proteins might be involved in non-target-site herbicide tolerance. However, to date, participation in non-target herbicide tolerance has been well established for only four gene families: P450s, GSTs, glycosyltransferases and ABC transporters (Yuan et al., 2007).

A wild sunflower (*Helianthus annuus* L. ssp. annuus) population (ANN-PUR) tolerant to imidazolinones (IMI) was discovered by Al-Khatib et al. (1998). The herbicide resistant trait was introgressed into elite inbred lines of sunflower by conventional breeding methods for the purpose of developing and deploying IMI-resistant cultivars known as Imisun sunflowers (Al-Khatib and Miller, 2000; Miller and Al-Khatib, 2002). Inheritance studies indicated that IMI tolerance in Imisun sunflowers is conferred by a partially dominant allele at the *Ahasl1* locus (*Imr1*, Bruniard & Miller, 2001; *Ar-pur*, Kolkman et al., 2004; or *Ahasl1-1*, Sala et al., 2008a). However, and in contrast with other examples reported in the literature for other crops (Tan et al., 2005), tolerance in Imisun sunflowers is also controlled by a second modifier gene (*Imr2*, also known as *"enhancer factor"*), which is present in some genetic backgrounds (Bruniard & Miller, 2001; Kolkman et al., 2004). Both genes should be present in homozygous condition in the final hybrid cultivar to achieve commercial levels of tolerance.

Previously, it was shown that *Ahasl1-1* harbors an A205V substitution conferring a target type tolerance (Kolkman et al., 2004). However, the nature of the tolerance endowed by *Imr2* has not been determined yet. The objectives of this work was to demonstrate that *Imr2* in Imisun sunflowers controls non-target site mechanisms of tolerance, and that P450 monooxygenases are one of such mechanisms.

MATERIALS AND METHODS

Two Imisun (*Ahasl1-1/Ahasl1-1,* IMI-1 and RHA426); two Clearfield Plus (*Ahasl1-3/Ahasl1-3;* R720 and B78) and one susceptible (*ahasl1/ahasl1*, HA89) inbred lines were used. IMI-1 is a $F_{2:6}$ maintainer line derived from the USDA Imisun-1 population with pedigree HA89*3/ANN-PUR (Al-Khatib and Miller, 2000). RHA426 is a restorer line selected from the USDA Imisun-2 population with pedigree RHA409//RHA376*2/ANN-PUR (Miller and Al-Khatib, 2002). R720 and B78, restorer and maintainer inbred lines respectively, are two CLPlus lines expressing the *Ahasl1-3* allele conferring high levels of IMI tolerance (Sala et al., 2008a). HA89 is a susceptible inbred line released by the USDA.

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) plants of each line were randomly assigned to two pre-treatments, one consisting in the application of 1 ml/L of malathion (diethyl 2-[(dimethoxyphosphorothioyl) sulfanyl] butanedioate, an inhibitor of P450 activity) and the other, used as control, consisting in the application of the same volume of water. Four hours after these initial pre-treatments, 10 plants of each pre-treatment were distributed into six treatments consisting in the application of increased doses of imazapyr (0; 40; 80; 160; 320; 640 g a.i. ha^{-1}, which corresponded to 0; 0.5x; 1x; 2x; 4x and 8x 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 arrangement of treatments in 10 replications. The experiment was replicated twice. 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 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.

Dose response curves were fitted for each genotype and replication, and the estimated value of the dose of herbicide needed to reduce the dry matter weight by the half (GR_{50}) was obtained. 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* = shoot 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 *GR50* 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 (R Development Core Team, 2011). Adequacy of model fit was determined by significance of the model approximate F-statistic and the coefficients of determination. Comparisons of the GR_{50} parameters among the Imisun genotypes were conducted by an analysis of variance using the model: GR_{50} = Inbred line + malathion effect + malathion × line interaction + error. Means were separated using Fisher's protected least significant difference (LSD) test at the 1 and 5% level of probability.

RESULTS

Susceptible plants died between 7 to 10 days after the application of any of the herbicide doses or malathion treatment. Both Clearfield Plus genotypes showed exactly the same response to increased doses of imazapyr application, with GR_{50} values greater than 1,000 (Table 1). No significant differences in GR_{50} values could be detected after the malathion treatment in these genotypes ($p>0.19$).

The log-logistic model accurately described biomass accumulation after imazapyr application for Imisun tolerant sunflower lines ($R^2>0.998$, Fig. 1). Genotype, malathion treatment, and their interaction significantly contributed to explain the observed variability in GR50 values for Imisun lines ($p<0.001$). Both genotypes showed striking differences in their response to increased doses of imazapyr (Fig. 1). As a matter of fact, GR_{50} estimate for RHA426 was twice times greater than the GR_{50} value of IMI-1 (Table 1).

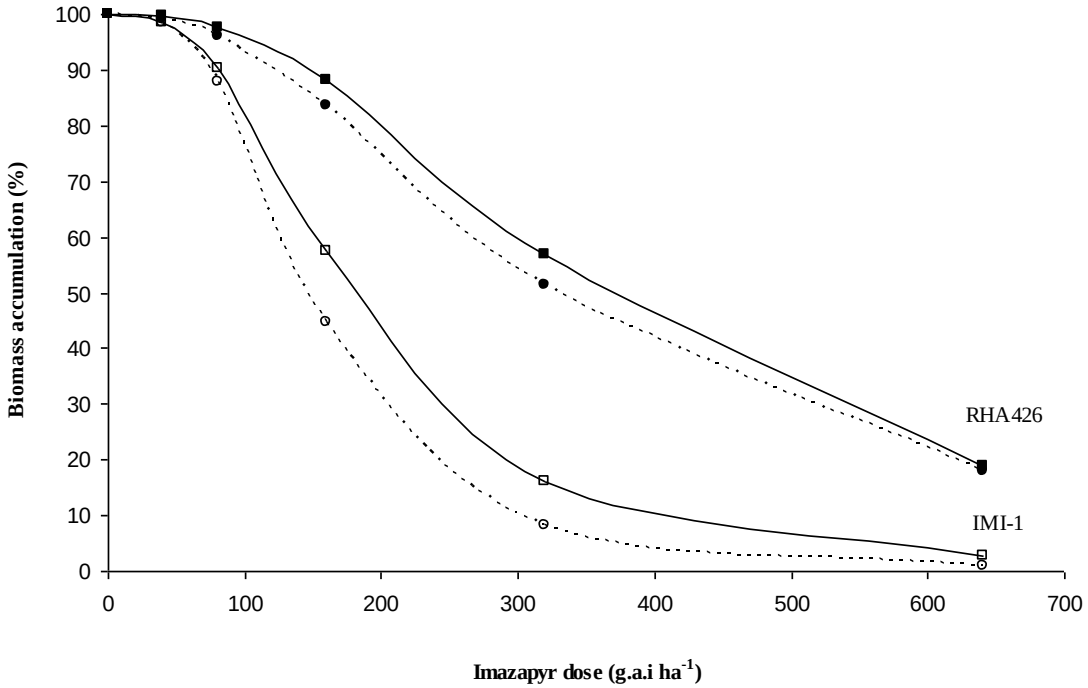

Fig. 1. Biomass accumulation of the Imisun lines, IMI-1 and RHA426 after 14 days of the application of different imazapyr doses, with or without malathion pretreatment (dashed and solid lines, respectively).

GR_{50} values for RHA426 ranged from 328.1 following malathion pretreatment to 357.2 without malathion application but they did not differ significantly ($P>0.068$, Table 1). Estimates of the doses of

imazapyr needed to reduce the biomass accumulation of the inbred line IMI-1 by the half (GR50) after malathion pretreatment (149.8 g a.i. ha^{-1}) was significantly lower ($p<0.01$) than the untreated control pretreated with water (177.8 g a.i. ha^{-1}). This reduction represented 15.7% of the GR50 value for this line.

Table 1. Estimates of the doses of imazapyr needed to reduce the biomass accumulation by the half (GR_{50}) following malathion (M1) or water (MO) pre-treatments for Imisun (IMI-1 and RHA 426) and CLPlus (B78 and R720) inbred lines. Percentage of reduction of GR_{50} due to malathion pre-treatment is also shown.

Inbred lines	Malathion pre-treatment	GR_{50}	[(GR_{50} M0-GR_{50} M1)/ GR_{50} M0]*100 (%)
IMI-1	M0	177.8	15.7
	M1	149.8	
RHA426	M0	357.2	8.2
	M1	328.1	
B78 CLPlus	M0	1359.4	-5.3
	M1	1431.9	
R720 CLPlus	M0	1716.8	7.6
	M1	1586.1	

DISCUSSION

Consistently with previous results, CLPlus lines showed a strong level of tolerance to increased doses of imazapyr. This high tolerance level was not modified by the inhibition of P450s by malathion. The same conclusion, but using a different approach, was reported previously (Sala et al., 2008b). In fact, the strong level of tolerance of CLPlus is not the result of the interaction of a target and a non-target site tolerance mechanism, since the original inbred line used to obtain the *Ahasl1-3* allele by EMS mutagenesis did not present an enhancer factor (Sala et al., 2008b).

Imisun lines used in this work (IMI-1 and RHA426) have the same genotype at the *Ahasl1* locus (*Ahasl1-1/Ahasl1-1*) and the same haplotype for the *Ahasl2* and *Ahasl3* loci (data not shown). Differences observed in their response to increased levels of imazapyr (GR_{50} values of 177.8 and 357.2) can only be explained by the presence of non-target mechanisms interacting with the target-site endowed tolerance. Moreover, this difference indicates that non-target mechanisms account for almost 50% of the GR_{50} values of both lines.

At the biochemical level, non-target mechanisms of tolerance in plants can include increased metabolism, sequestration, reduced uptake and/or translocation of the herbicide (Powles and Holtum 1994). For AHAS inhibitor resistance herbicides (AIH) both target site and non target site resistance mechanisms have been previously reported (Saari et al., 1994). In fact, it was demonstrated that IMI-tolerant sunflowers showed less ^{14}C-herbicide absorption and translocation than susceptible genotypes (White et al., 2002).

Our results indicate that one of the putative mechanisms of non target tolerance ―the inhibition of P450 activity― is active in one of the Imisun lines analyzed, IMI-1. Even in this case, this inhibition only explained 15% of the GR_{50} value observed in this line. P450 monooxygenases or mixed function oxidases carried out oxidations, a typical detoxification reaction in plants (Werck-Reichhart et al., 2000; Schuler and Werck-Reichhart, 2003; Yun et al., 2005). The variety of reactions mediated by P450s (Schuler and Werck-Reichhart, 2003; Inui and Ohkawa, 2005) results in oxygenated products that are normally more reactive or more soluble, thus setting the stage for subsequent detoxification reactions (Morant et al., 2003). The non-target site component of tolerance of the type found in Imisun sunflowers was isolated, evaluated and characterized independently from the AHAS system by screening inbred wild type lines with imazamox and malathion (Kaspar et al., 2011).

Nevertheless, the obtained results indicated that other mechanisms, apart from P450 activity, seem to be of greater importance in controlling the tolerance in Imisun sunflowers. Hence, the genetic control of this trait seems to be more complex than already postulated. It must be considered that plant detoxification processes endowing AIH resistance may be visualized as a four-step schema which include oxidation, conjugation, transport and degradation of the herbicide molecule (Martinoia et al., 1993; Sandermann, 2004; Bartholomew et al., 2002). P450 activity is only one of these components that act

cooperatively to endow the tolerance. It can be concluded that the previously described gene *Imr*$_2$ (*E*, or *enhancer*) can control P450 activity in certain cases but that other non-target mechanisms should be present to achieve adequate levels of tolerance in Imisun sunflowers, as those explaining the differences in tolerance observed between IMI-1 and RHA426. In fact, *Imr2* was postulated as the result of the genetic analyses of only one cross, namely HA89/HA425 (Bruniard and Miller, 2001). It is likely that other genetic backgrounds of sunflower present other types of non-target site mechanisms of tolerance. In fact, several physiological mechanisms might be involved in the non-target site component of tolerance in Imisun sunflowers. This physiological polymorphism permit to explain the variability for crop injury observed among sunflower hybrids carrying the *Ahasl1-1* allele in homozygous state when challenged with imidazolinones under the same environmental conditions.

The presence of target and non-target site mechanisms endowing tolerance to AIH is not exclusive for Imisun sunflowers. In fact, it was reported that differences in crop injury among sulfonylurea-tolerant breeding lines (*Ahasl-2/Ahasl1-2*) is also the result of the presence of modifier genes (Miller and Zollinger, 2004).

Imisun sunflowers represent the first example of a commercial herbicide tolerance trait conferred by two independent tolerance mechanisms (Tan et al., 2005). Perhaps the intermediate level of tolerance endowed by the target type tolerance (A205V substitution) permitted to identify the second system. On the other hand, the rather complex nature of the non target tolerance mechanisms involved in Imisun tolerance permits also to explain the difficulties arising when converting conventional lines to their tolerant counterparts using solely a molecular marker based approach.

The insecticide malathion belongs to the group of organophosphates. When oxygenated by P450s, it releases atomic sulphur that covalently binds to the apoprotein, leading to its inactivation. When crops are treated simultaneously with an herbicide and an insecticide that are recognized by the same P450, it could lead to crop destruction. (Werck-Reichhart et al., 2000). For this reason, caution should be taken to avoid the application on Imisun sunflowers of an organophosphate insecticide together with imidazolinones.

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