Effect of cytochrome P450s inhibitors on imidazolinone resistance in sunflower

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ABSTRACT

• Imidazolinone resistance genes have been introgressed from a sunflower wild population (PUR *H. annuus L.* collected in Kansas) to elite inbred lines. A digenic model has been proposed as the genetic basis of the inheritance of imidazolinone resistance. This model assumes that both parents must express two resistance genes, the major semidominant gene Imr_1 and the modifier Imr_2, to achieve complete resistance in the hybrids. Imr_1 is an allelic variant of the *AHAS1 locus* that codes for the acetohydroxyacid synthase catalytic subunit. The effect of the modifier *locus*, Imr_2, remains unknown and it could be related to non-target-site resistance. Cytochrome P450s-mediated herbicide metabolism has been identified as being responsible for the resistance to AHAS inhibitors in several species. The objective of this study was to evaluate the growth response to imazapyr in combination with P450s inhibitors in sunflower plantlets.

• Two sunflower inbred lines were used in this study: HA425 and HA89, classified as resistant ($Imr_1Imr_1Imr_2Imr_2$) and susceptible ($imr_1imr_1imr_2imr_2$) respectively. Seeds were placed in plastic pots filled with commercial perlite and watered by capillarity with nutritive solution. After 7 days of incubation under controlled conditions plants were treated with 0 (control), 1, 10 and 100 µM imazapyr alone and in combination with P450s inhibitor. Two P450s inhibitors were assayed in independent experiments: piperonyl butoxide (PBO) and 1-aminobenzotriazole (ABT). After 15 days plantlets were collected and images were obtained by a scanner. Primary root length (RL) and the longest lateral root length (LRL) were measured from scanned images using ImageJ 1.44. Tomato Analyzer 3.0 software was used to measure shoot length (SL), foliar area (FA), and color of the first pair of leaves. The leaf color was estimated through the hue angle parameter. Three replications were done in each experiment and each replication consisted of 10 plants (total number of plants=960). Data were analyzed by two-way analysis of variance.

• The reduction of hue angle with increasing doses of herbicide was associated with the appearance of leaf symptoms such as chlorosis and necrosis. The effect of imazapyr was significant ($p<0.05$) in both lines for all the evaluated variables but the resistant line decreased its growth at higher herbicide doses. The interaction between the effects of imazapyr and P450s inhibitors on LRL was significant ($p<0.05$) only for the resistant line. Furthermore, a significant interaction between the effects of imazapyr and PBO ($p<0.05$) was observed on FA in the resistant line. The largest differences in LRL and FA between absence and presence of P450s inhibitor occurred at the intermediate dose of herbicide (10 µM imazapyr).

• The increased phytotoxicity of imazapyr in the resistant line when P450s inhibitor was present is aligning with suggestions previously made that P450s mediates a detoxification mechanism and contributes to herbicide resistance. This mechanism could be related to the effect of the modifier gene Imr_2.

• This study contributes to understanding the mechanisms of imidazolinone resistance in this species. These results encourage further experimentation on the molecular and biochemical levels to asses the role of P450s in endowing herbicide resistance.

Key words: imazapyr - piperonyl butoxide - 1-aminobenzotriazole - herbicide resistance- root growth - shoot growth

INTRODUCTION

Imidazolinone herbicides control a wide spectrum of grass and broadleaf weeds at low application rates and have favorable environmental properties compared to other chemistries (Tan et al., 2005). These herbicides are specific and potent inhibitors of acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS), which is the first common enzyme in the biosynthetic pathway of branched-chain amino acid (Shaner et al., 1984).

In sunflower, imidazolinone resistance genes from a wild population (PUR *H. annuus* collected in Kansas) have been introgressed to elite germplasm (Al-Khatib et al., 1998; Miller and Al-Khatib, 2002). A digenic model has been proposed as the genetic basis of the inheritance of this trait. This model assumes that both parents express two resistance genes, the major semidominant gene Imr_1 and the modifier Imr_2, to achieve complete resistance in the hybrids (Bruniard and Miller, 2001). Imr_1 is an allelic variant of the *AHAS1 locus* that codes for the acetohydroxyacid synthase catalytic subunit (Kolkman et al., 2004). The effect of the modifier *locus*, Imr_2, remains unknown and it could be related to non-target-site resistance.

Non-target-site resistance comprises a wide range of mechanisms that act to minimize the amount of herbicide reaching the target site. As examples these mechanisms include: decreasing the amount of herbicide penetration into the plant, decreasing the herbicide translocation rates, and increasing the herbicide metabolic rates (Powles and Yu, 2010). Herbicide can be detoxified by plants in a process that follows a four-phase scheme. In phase I, herbicide molecules are activated so that certain functional groups can be exposed for phase II conjugation enzymes. Phase II detoxification generally involves conjugation of a sulky hydrophilic molecule to the activated xenobiotic, which enables the end product of phase II detoxification to be recognized by the phase III transporters. Phase III detoxification involves transporting the conjugated molecule into the vacuole or extracellular space by active transport. Finally, phase IV detoxification involves further degradation of the conjugated molecule in the vacuole or extracellular spaces (Yuan et al., 2007).

Cytochrome P450 monooxygenases (P450s) belong to the most important phase I enzymatic system involved in herbicide metabolism by weeds and crops (Siminsky, 2006). P450s-mediated herbicide metabolism has been identified as being responsible for the resistance to AHAS inhibitors in several species (Powles and Yu, 2010). Because the biochemical characterization is difficult, evidence for P450s involvement has been provided mainly by *in vivo* experiments using P450s inhibitors such as tetcyclasis, 1-aminobenzotriazole (ABT), piperonyl butoxide (PBO), and certain organophosphate insecticides (Werch-Reichhart et al., 2000). Different patterns of herbicide inhibition by P450s inhibitors *in vivo* were observed. These differences were mainly due to different P450s isoenzymes involved in enhanced herbicide detoxification (Preston et al., 1996; Letouzé and Gasquez, 2003).

The aim of this study was to determine if the herbicide detoxification mechanism catalysed by P450s contributes to the imidazolinone resistance in sunflower. For this purpose, we evaluated the growth response to imazapyr in combination with P450s inhibitors in sunflower plantlets.

MATERIALS AND METHODS

Two sunflower inbred lines were used in this study: HA425 and HA89, classified as resistant (Imr_1 $Imr_1Imr_2Imr_2$) and susceptible (imr_1 $imr_1imr_2imr_2$) respectively (Bruniard and Miller, 2001).

Plants were grown in plastic pots (4 cm wide, 5.5 cm tall) filled with commercial perlite. Pots were placed in plastic trays and watered by capillarity with a nutritive solution consisting of Murashige and Skoog's (1962) medium (1.1 g/l). Pots were incubated at 25 ± 2 ºC with a 12 h photoperiod (100 µmol/m^2/s). After 7 days plants were treated with 0 (control), 1, 10 and 100 µM imazapyr alone and in combination with one of two different P450s inhibitors: piperonyl butoxide (PBO, 50 µM) and 1-aminobenzotriazole (ABT, 70 µM). Preliminary assays were used to determine the maximum levels of each inhibitor to be used without phytotoxic effects in the absence of herbicide (data not shown). Each P450s inhibitor was added to the nutrient solution 24 h before herbicide application. After 15 days, plantlets were collected and their roots washed to remove perlite debris. Plants were dissected and images were obtained and recorded. Primary root length (RL) and the longest lateral root length (LRL) were measured from scanned images using ImageJ version 1.44 (Abramoff et al., 2004). Shoot length (SL), foliar area (FA), and color of the first pair of leaves were evaluated using the Tomato Analyzer 3.0 software (Rodríguez et al., 2010). The leaf color was estimated through the hue angle parameter. The experimental design was a randomized block design with three replications. Each replication consisted of 10 plants (total number of plants = 960).

For each experiment, data was analyzed by two-way analysis of variance. Normality of the empirical distribution of each variable was assessed by the Shapiro-Wilks W-test statistic. Homogeneity of variance was evaluated using Bartlett's test. Within a genotype, means were compared using Tukey's multiple comparison test. Statistical analyses were performed using R software, version 2.12.1 (R Development Core Team, 2010).

RESULTS

P450s inhibitors applied alone had no detectable phytotoxic effect for any genotype tested ($p>0.05$). The effect of imazapyr was significant ($p<0.05$) in both lines for all the evaluated variables except for shoot length measured in the ABT experiment for the resistant genotype. The resistant line decreased its growth at higher herbicide doses (Fig. 1-2).

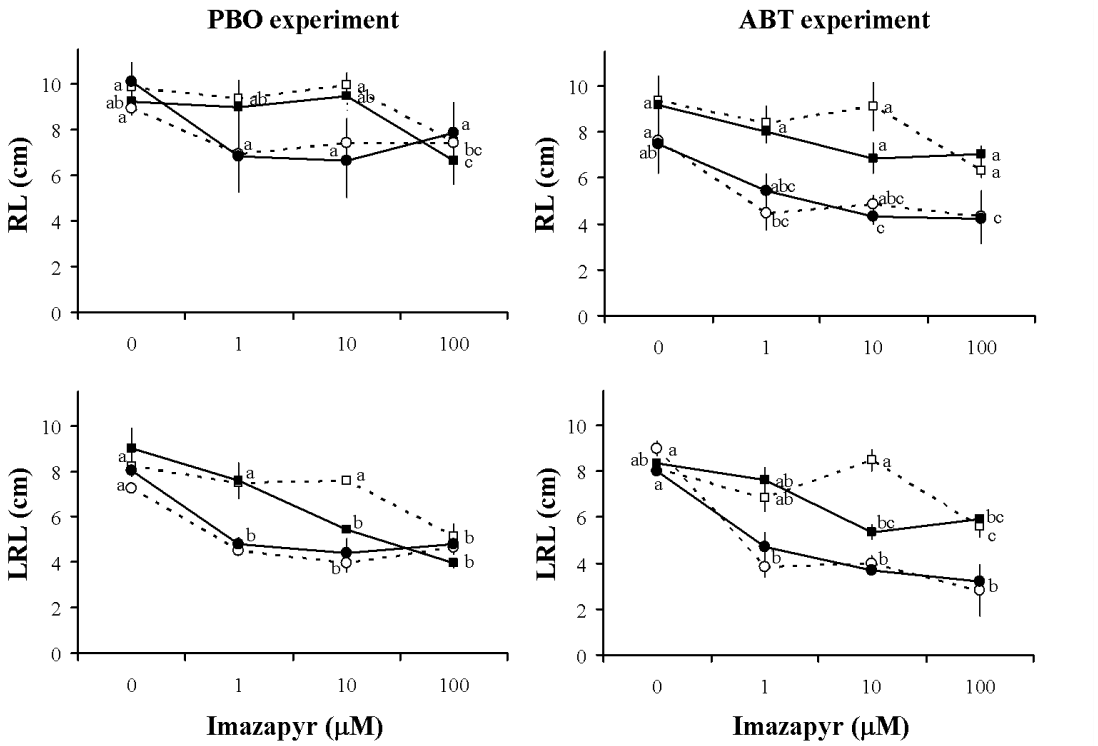

Fig 1. Root growth response to imazapyr in combination with P450s inhibitors in sunflower plantlets. Primary root length (RL), and the longest lateral root length (LRL) were measured in PBO experiment and ABT experiment. Resistant line HA425 in presence (□□■□□) or absence (---□---) of P450s inhibitor. Susceptible line HA89 in presence (□□●□□) or absence (---○---) of P450 inhibitor. Vertical bars represent standard errors of the mean (3 replicates). Within a genotype, means with the different letter are significantly different ($\alpha=0.05$).

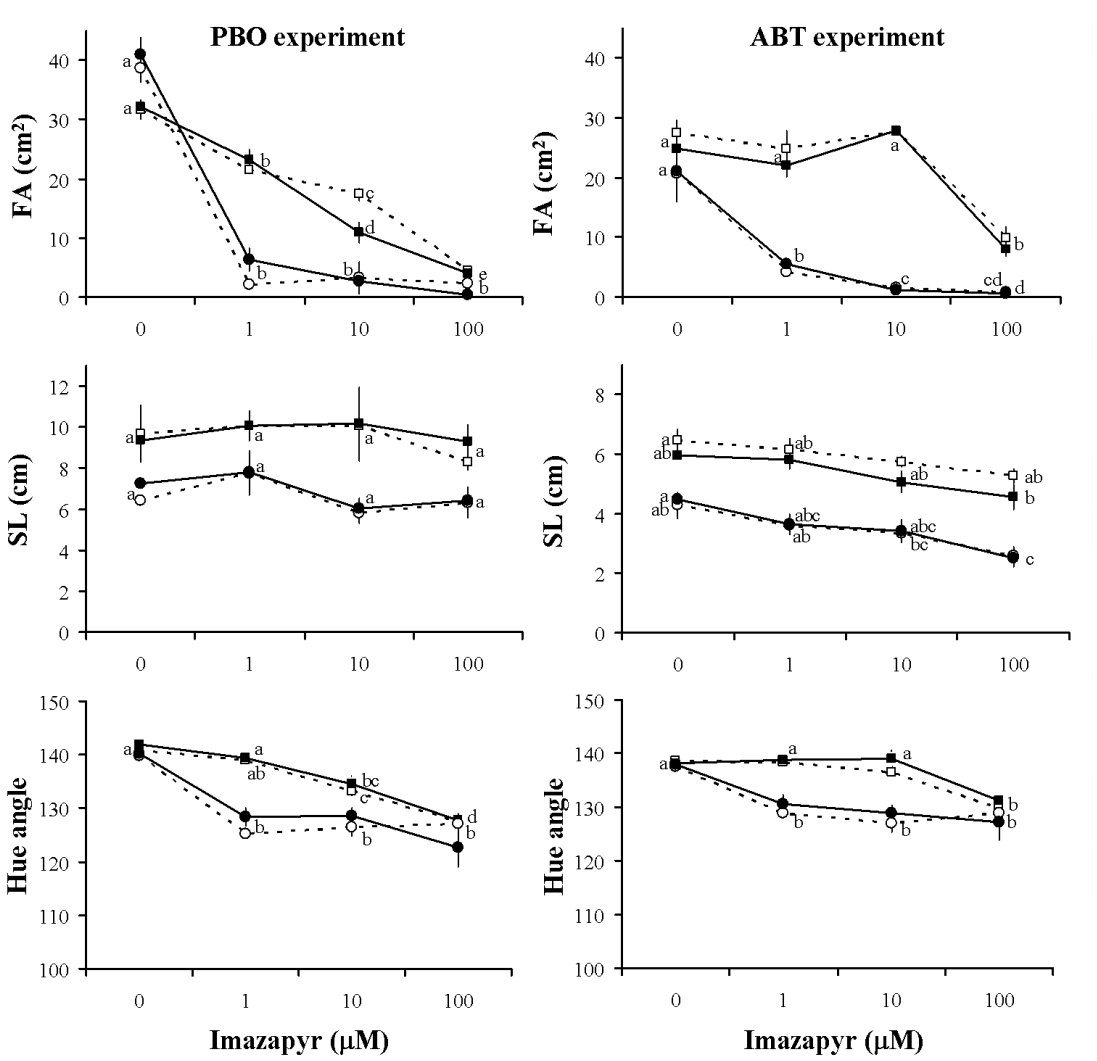

Fig 2. Aerial growth response to imazapyr in combination with P450s inhibitors in sunflower plantlets. Foliar area (FA), shoot length (SL), and color of the first pair of leaves (Hue angle) were measured in PBO experiment and ABT experiment. Resistant line HA425 in presence (□□■□□) or absence (---□---) of P450s inhibitor. Susceptible line HA89 in presence (□□●□□) or absence (---○---) of P450 inhibitor. Vertical bars represent standard errors of the mean (3 replicates). Within a genotype, means with the different letter are significantly different (α=0.05).

The interaction between the effects of imazapyr and P450s inhibitor was significant ($p<0.05$) for the lateral root growth in both experiments (PBO and ABT) only for the resistant genotype. Moreover, there was an interactive effect on the foliar area caused by PBO and imazapyr in the resistant genotype ($p<0.05$). In all these cases, when imazapyr*P450s inhibitor interaction was present, significant differences between presence and absence of P450s inhibitor were observed for 10 µM imazapyr (Fig. 1-2).

The reduction of hue angle with increasing herbicide concentrations was associated with the appearance of leaf symptoms such as chlorosis and necrosis. The appearance of these symptoms was observed in both resistant and susceptible lines; however, the damage in the later was greater and visible at low doses compared to the resistant line (Fig. 2).

DISCUSSION

Most of the traits measured in this study were already described as useful parameters for the selection of sunflower genotypes differing in imidazolinone resistance (Breccia et al., 2011). In this study we

demonstrated that hue angle is a useful trait for discriminating resistant from susceptible genotypes when exposed to 1-10 μM imazapyr in early herbicide screening (Fig. 2). Leaf color is often measured with visual scales which are not quantitatively rigorous. As demonstrated here, digital image analysis can be considered as way to phenotype color traits of vegetables and fruit crops more precisely. Tomato Analyzer software program provided an objective and reliable evaluation of morphology and color variation of plant organs (Rodríguez et al., 2010).

P450s genes are known to be involved in imidazolinone metabolism (Manabe et al., 2007). The involvement of P450s in herbicide detoxification has been demonstrated using *in vivo* approaches for several species. The reduction of herbicide resistance by PBO has been reported for AHAS inhibitors in *Phalaris minor* (Singh et al., 1998), *Echinochloa phyllopogon* (Fischer et al., 2000), *Zea mays* and *Glycine max* (Kwon et al., 1995). ABT was also employed to study *in vivo* P450s involvement in herbicide resistance (Christopher et al., 1994; Bravin et al., 2000). Numerous studies demonstrated that the effects of P450s inhibitors evaluated at whole plant level correlated well with their biochemical ability to inhibit herbicide metabolism (Preston et al., 1996)

In this study, P450s inhibitors increased imazapyr toxicity only for the resistant genotype. Both PBO and ABT caused a significant reduction in LRL in the presence of 10 μM imazapyr, but the significant reduction in FA was observed only for PBO (Fig. 1-2). The differential response of ABT and PBO may be explained by the fact that P450s isoforms have different inhibitor specificity and that isozymes are differently expressed between tissues (Siminsky, 2006). Similar results were reported for a sunflower line with multiple herbicide tolerance reversed by malathion (Kaspar et al., 2011). In that case, the P450s inhibitor only increased herbicide toxicity when measured as inhibition of shoot length but no as root length.

The increase in phytotoxicity of imazapyr induced by P450s inhibitors was observed only for the resistant genotype in the presence of 10 μM imazapyr. These results suggest that P450s mediates a detoxification mechanism and contributes to herbicide resistance in this species. The reduction of growth parameters caused by P450s inhibitors plus imazapyr in the resistant line was still not as low as those of the susceptible genotype treated with herbicide (Fig. 1-2). These results are consistent with the action of a modifier gene that partially affects herbicide resistance. Even though P450s activity was inhibited, the resistant genotype also possesses an insensitive AHAS isoform (Imr_1 major gene) that allowed a higher level of resistance than susceptible genotype. The results obtained in this study suggest that Imr_2 could be related to a mechanism of herbicide detoxification catalysed by P450s.

The mechanism of acquiring increased metabolism could be related to a duplication of P450s genes, although a point-mutation in a structural gene is also possible. Another plausible explanation is the altered regulation of a stress-response pathway, in which one or more P450s are induced, leading to a constitutive overexpression. Further analysis including additional genotypes, molecular genetic and biochemical experiments should be conducted to completely understand the molecular basis of Imr_2 action.

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