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Absorption and translocation of imazethapyr as a mechanism responsible for resistance of *Euphorbia heterophylla* L. biotypes to acetolactate synthase (ALS) inhibitors

Absorción y translocación de imazetapir como mecanismo responsable de la resistencia a inhibidores de la acetolactato sintasa (ALS) en biotipos de *Euphorbia heterophylla* L.

Guido A. Plaza¹, María Dolores Osuna², Rafael De Prado³ and Antonio Heredia⁴

Abstract: The effect of weeds on reduction of agricultural production is estimated between 30% and 50%. Imazethapyr is a herbicide of imidazolinone group that inhibits activity of enzyme acetolactate synthase (ALS), the first common enzyme in the biosynthetic pathway of valine, leucine, and isoleucine. *Euphorbia heterophylla* is common specie in soybean fields of Brazil. The study reports about a population of *Euphorbia heterophylla* resistant to imazethapyr. The objectives of the present work were to quantify the level of sensitivity to this herbicide in imazethapyr-resistant and -susceptible *E. heterophylla* populations evaluate the role of differential penetration into leaves as determining plant resistance to imazethapyr, and compare the waxy cells of R and S populations. The R population had a lower penetration rate compared with that of S population during the six first hours of incubation with the herbicide. Further studies indicated that R population was not different from S population in terms of translocation, metabolism, or target site (ALS enzyme) of imazethapyr action. Analysis of the leaf cuticle surface by scanning electron microscopy revealed higher wax density in the leaf cuticles of population R than that in S population. Thus, it is suggested that R population is resistant to imazethapyr because increased wax content of its cuticle permits less penetration of herbicide into the plant.

Additional key words: resistant mechanism, herbicides, wild poinsettia

Resumen: El efecto de las malas hierbas en la disminución de la producción agrícola está considerado entre 30% y 50%. Imazetapir es un herbicida que actúa sobre la enzima acetolactato sintasa (ALS), primera enzima común en la ruta biosintética de la valina, leucina e isoleucina. *Euphorbia heterophylla* es una especie común en los campos de soya del Brasil. Actualmente se reporta una población resistente a imazetapir, herbicida perteneciente al grupo de las imidazolinonas. El objetivo de los ensayos de absorción y translocación fue estudiar las posibles diferencias de penetración foliar y movimiento del ¹⁴C-imazetapir en dos biotipos de *E. heterophylla* L. En el biotipo resistente, se registró una menor absorción durante las primeras 6 h después del tratamiento, tendencia que se diluye en los siguientes tiempos de evaluación. Las tendencias de los valores de translocación fueron similares durante las evaluaciones realizadas. Los resultados de los análisis de química de ceras no arrojaron diferencias entre la composición cuticular entre los biotipos; sin embargo, los estudios de microscopía electrónica de la hoja sí muestran diferencias en la morfología y la cantidad de ceras cuniculares, factores que determinan el comportamiento resistente del biotipo R.

Palabras claves adicionales: mecanismos de resistencia, herbicidas, lecherón

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Introduction

IMAZETHAPYR IS A BROAD-SPECTRUM imidazolinone herbicide, absorbed by the foliage and roots with rapid translocation in the xylem and phloem to the meristematic regions where it accumulates. The mode of action of imazethapyr is the inhibition of acetolactate synthase (ALS) (Shaner *et al.*, 1984), the first common enzyme in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Saari and Mauvais, 1996). That enzyme is the site of action of four other herbicide classes, namely sulfonylureas, triazolopyrimidines, pyrimidinyl thiobenzoates, and sulfonylaminocarbonyl-triazolinones (Hawkes, 1989; Hawkes *et al.*, 1989; Kishore and Shah, 1988; Heap *et al.*, 2006).

Resistance to ALS-inhibiting herbicides has become a major concern in many crops around the world, and has increased in recent years. In most cases, resistance was due to an alteration of the target ALS enzyme that renders it much less sensitive to herbicide inhibition than that of susceptible biotypes (Devine and Eberlein, 1997). There are also a few examples where a lack of herbicide absorption or translocation to the site of action contributes to ALS herbicide resistance. These potential mechanisms usually play no role or have only a secondary role compared with metabolic inactivation or target site insensitivity.

Wild poinsettia (*Euphorbia heterophylla* L.) is a dicotyledon weed in the Euphorbiaceae family and it is an important weed found in soybean field production in Brazil. This weed evolved resistance to group B/2 herbicides (ALS-herbicides) in 1992 under these conditions (Vidal, 1997). The imazethapyr-resistant mechanism has not been established for *E. heterophylla*. The objectives of this work were to quantify the level of sensitivity of imazethapyr-resistant (R) and susceptible (S). *E. heterophylla* populations to this herbicide, evaluate the role of differential penetration into leaves as a determinant of resistance to imazethapyr, and compare the waxy cells of R and S populations.

Materials and methods

Absorption and translocation

[¹⁴C]imazethapyr was mixed with commercial imazethapyr to prepare an emulsion (20,66 $\mu\text{Ci} \cdot \text{mg}^{-1}$ specific activity) with an imazethapyr concentration corresponding to a 100 g a.i. $\cdot \text{ha}^{-1}$ treatment with a 200

L $\cdot \text{ha}^{-1}$ volume. The commercial herbicide was added in order to provide the necessary additives to the mix to simulate a field treatment. This emulsion containing labeled herbicide was applied to the leaf surface of each biotype in four 0.5 μL droplets using a microapplicator (Hamilton PB 6000 Dispenser, Hamilton Co., Reno, NV).

For absorption and translocation study, plants were harvested in batches of 3 plants at 6, 12, 24, and 48 h after herbicide application and separated into treated leave, upper and under foliage of treated leave and roots. The surface of the treated leaves was washed with 3 mL 80% methanol. Washes from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA). The washed treated zone and the other plant sections were dried (60 °C for 48 h) and combusted in a sample oxidizer (Packard 307). The ¹⁴CO₂ released was trapped in 15 mL of Permafluor+Carbosorb (2+1, v/v) (Packard Instruments Co.) and radioactivity was quantified by liquid scintillation counting. Absorbed imazethapyr was defined as the sum of the radioactivity in all plant sections. Recovered radioactivity was defined as absorbed radioactivity plus radioactivity in the leaf washes. Percentage absorption was defined as: (absorbed radioactivity/recovered radioactivity) $\times$ 100, whereas percentage radioactivity translocated to individual plants sections was estimated as (radioactivity in a plant section/absorbed radioactivity) $\times$ 100. The assays were repeated twice. Qualitative studies of translocation were made by removing plants from pots at the same intervals after herbicide treatment. Roots were washed and plants were blotted dry, pressed against a x-ray film (Agfa-Curix), and stored at -4 °C. The film was developed after 3 weeks of exposure.

Analyses of epicuticular waxes

The wax extraction procedure was previously described by Elmore and Paul (1998). Epicuticular waxes were extracted from several leaves of different biotypes. These leaves were rinsed with chloroform for 30 s at room temperature. The resulting solution was filtered through analytical-grade filter paper and evaporated under N₂. The residue obtained was rediluted in 500 μL of chloroform and then subjected to thin layer chromatography, using silica gel plates and 100% benzene as the mobile phase. Cuticular waxes constituents were separated into different compound classes that were determined by R_f values, as described by Hamilton (1995).

Surface morphology studies by scanning electron microscopy

Plants of two biotypes were grown under controlled environmental conditions. Plants with three to four leaf growth stage were used in this experiment. Samples of about 1 cm² cut from the middle of the leaf lamina of young, fully developed leaves (adaxial and abaxial surface) were affixed to aluminium stubs. All specimens were sputter coated to prevent charging effects that would distort the electric fields in the electron microscope and were examined in a scanning electron microscope at 15 Kv and photographed.

Results and discussion

Plaza *et al.* (2003) reported the resistance factor for the biotype R 5.52 when measured directly in plant, and 0.44 corresponding to enzymatic activity of ALS.

The percentage of absorption in R biotype was smaller than that in S biotype during the first evaluation and this tendency remained constant during the experiment. The moments of more product penetration corresponded to the moments of higher concentration of the product in the external part of the leaf with important differences among the biotype (table 1).

Table 1. Absorption and translocation of ¹⁴C-imazethapyr in a S and R biotypes of *Euphorbia heterophylla* L.

Biotypes	Time (h)	¹⁴ C (% of recovered)				
		Applied radioactivity*	Upper foliage	Treated leave	Under foliage	Roots
R	6	28.61	3.67	85.23	6.65	4.45
	12	70.53	6.12	72.46	8.64	12.78
	24	76.37	12.06	56.83	12.01	19.10
	48	60.89	38.54	23.00	16.87	21.58
S	6	73.57	2.77	88.42	4.78	4.04
	12	77.81	7.74	78.93	5.17	8.16
	24	92.71	16.21	59.68	8.60	15.51
	48	83.69	31.38	33.30	10.99	24.33

* Data represent the means of three replicates.

The translocation process of the herbicide inside the plant did not differ among two biotypes, and may not explain the resistant behavior of R biotype (table 1). The autoradiography elucidated the same tendency with no differences among the biotypes (figure 1).

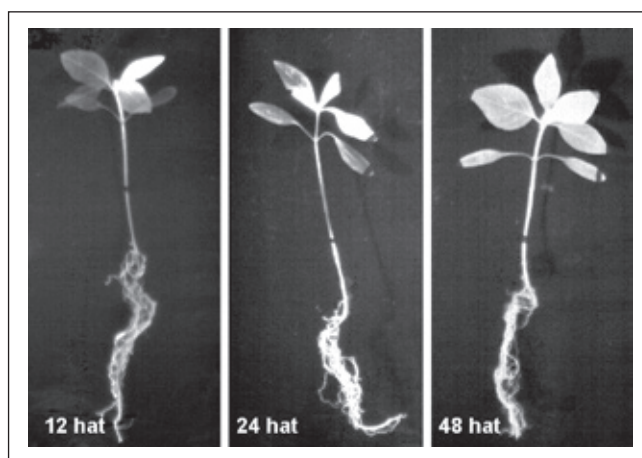


Figure 1. Autoradiography of *Euphorbia heterophylla* L. resistant biotypes (R) to imazethapyr at different times, 12, 24 and 48 h after treatments (hat). The places of more light intensity represent the location of the ¹⁴C-imazethapyr.

According to Hamilton (1996), the main constituents of epicuticular waxes are *n*-alkane of long chain (R_f 0.91), esters of long chain (R_f 0.71), and alcohols of long chain (R_f 0.15). The results demonstrate a common composition of epicuticular waxes in both biotypes that explains the lack of difference in wax composition or absorption patterns among two biotypes (figure 2). The studies of the external part of the cuticle allowed estimating heaps of epicuticular waxes that were presented morphologically in form of small sheets. The quantities

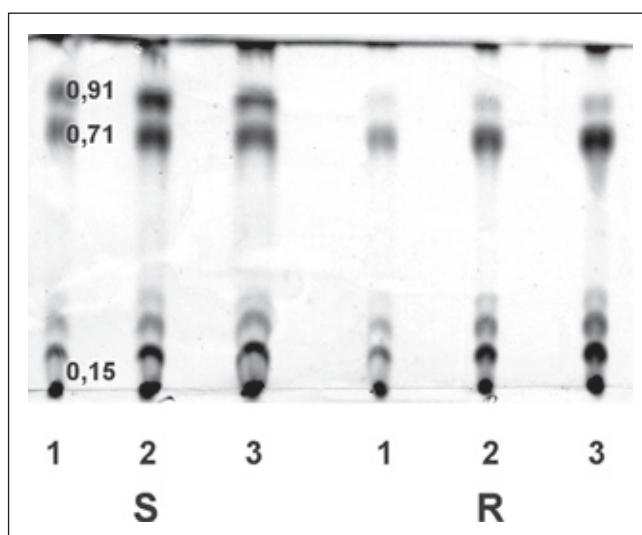


Figure 2. Waxes identification epicuticulars in biotypes R and S of *Euphorbia heterophylla* L. by means of thin layer chromatography, using benzene like mobile phase. The concentrations of waxes were: rail 1, a volume; rail 2, two volumes and rail 3, four volumes.

of epicuticular waxes varied among the biotypes (figure 3) with higher density per unit of surface in biotype R and smaller density in the susceptible biotype.

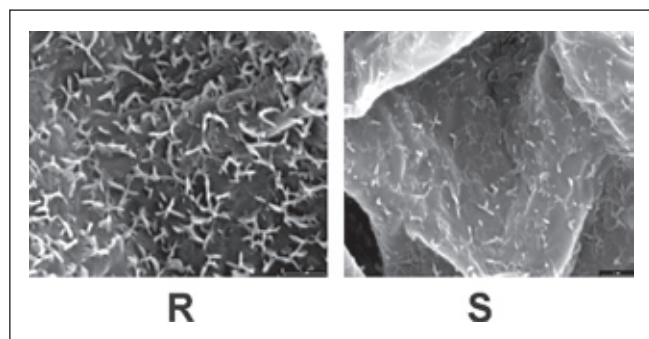


Figure 3. Scanning electron microscopy of leaf surface to 14.000 increases of the surface adaxial of the biotypes R and S of *Euphorbia heterophylla* L.

The results allowed clarifying the role of waxes as a barrier to penetration of herbicide imazethapyr, understanding the differences in absorption values among the biotypes, and explaining resistance of biotype R.

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