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Lack of Cross-Resistance of Imidazolinone-Resistant Cell Lines of *Datura innoxia* P. Mill. to Chlorsulfuron[1]

Evidence for Separable Sites of Action on the Target Enzyme

Praveen K. Saxena* and John King

Department of Horticultural Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (P.K.S.), and Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OWO (J.K.)

ABSTRACT

Two cell lines of *Datura innoxia* resistant to two imidazolinone herbicides, imazapyr and imazaquin, were isolated from mutagenized, predominantly haploid cell suspension cultures. Both of the resistant variants were >1000-fold more resistant than the wild-type to the two imidazolinones. The variant resistant to imazapyr showed cross-resistance to imazaquin and vice versa, but no cross-resistance to a structurally different inhibitor, chlorsulfuron, a sulfonylurea herbicide, was observed. The target enzyme, acetolactate synthase, extracted from imidazolinone-resistant cell lines was not inhibited by imazapyr or imazaquin but was sensitive to chlorsulfuron indicating separable sites of action for these inhibitors. The variation in resistance and cross-resistance of chlorsulfuron-resistant (PK Saxena, J King [1988] Plant Physiol 86: 863–867) and imidazolinone-resistant cell lines of *Datura innoxia* demonstrates the possibility of separate mutations of acetolactate synthase gene resulting in specific phenotypes.

Introduction of herbicide resistance into cultivated species is considered an important application of plant biotechnological research (3, 12). Although a number of inhibitors belonging to chemically different classes of compounds have been identified and developed as commercial herbicides, the precise mechanisms of their action have not been elucidated. Sulfonylureas and imidazolinones herbicides, however, have been at the center of research in this regard because they cause observable suppression of plant growth by blocking cell division and the mode of their action involves the inhibition of an important and relatively well defined biochemical pathway. The pathway in question leads to the biosynthesis of the essential branched chain amino acids, valine, leucine, and isoleucine (1, 14–16, 19), and the site of action of the herbicides is the enzyme ALS[2] (acetohydroxyacid synthase; EC 4.1.3.18), the first, and principal, enzyme in the biosynthesis of these amino acids (4, 13, 19).

The inhibition of the activity of ALS by sulfonylureas and imidazolinones leads to the logical assumption of a common site of action for these herbicides on the ALS molecule. In our previous study (17), we isolated a number of *Datura innoxia* cell lines which were resistant to CS, a sulfonylurea herbicide, and checked their cross-resistance to imidazolinones. Surprisingly, some of the CS-resistant variants had either no cross-resistance to imidazolinones or were even more sensitive to them than the wild-type parent. This observation suggested the possibility of separable functional (or even structural) sites of action on ALS for sulfonylureas and imidazolinones. In this communication we report the lack of cross-resistance of imidazolinone-resistant cell lines to CS, thus strengthening the hypothesis that the ALS molecule indeed has separable sites of action for these two classes of inhibitors.

MATERIALS AND METHODS

Cell Suspension Cultures

Cell suspension cultures of *Datura innoxia* P. Mill. were maintained in a modified B5 medium, hereafter referred to as B5A, containing the ingredients of B5 formulation (8), 1 $mg \cdot L^{-1}$ 2,4-D and 500 $mg \cdot L^{-1}$ ammonium nitrate. The wild-type cell line consisted of haploid cells, predominantly (76–84%). All cell lines were subcultured at weekly intervals by transferring 5 mL of cell suspension from a 1-week-old flask to 50 mL of fresh medium dispensed in a 250 mL DeLong flask. Cell suspension cultures were maintained at 25°C under continuous illumination of about 5 $\mu E \cdot m^{-2}s^{-1}$ (provided by cool-white fluorescent tubes) on a horizontal gyratory shaker (150 rpm).

Isolation of Resistant Cell Lines

The method of isolating resistant cell lines was modified from our previous protocol (17). The wild-type cell suspension was filtered through a 500-μm nylon screen, and the cells were collected over Miracloth. After washing once with B5A medium, the cells were plated at a cell density of 10^3 cell

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[2] Abbreviations: ALS, acetolactate synthase; CS, chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazine-2-yl) aminocarbonyl] benzenesulfonamide); imazapyr, isopropylamine salt of 2-[4,5-dihydro- 4-methyl- 4-(1-methyl-ethyl)-5-OXO -1H-imidazol-2-yl]-3-pyridinne carboxylic acid; imazaquin, 2-[4,5-dihydro-4-methyl-4-(1-methyl-ethyl)-5-OXO-1H-imidazol-2-yl]-3-quinoliine carboxylic acid.

colony forming units per mL; a colony forming unit consisted of 1 to 6 cells. The growth of developing cell colonies was monitored by microscopic examination, and cell colonies originating from 1 to 4 cells were isolated, subcultured, and maintained as clonal cell lines. One such wild-type clonal cell line was used for the isolation of resistant variants. For mutagenization of cells, a filter-sterilized solution of ethyl methane sulfonate (0.2% final concentration, v/v) was added to the culture flask and the flasks returned to the shaker. Following a 2.5 h treatment, the cells were washed 4 to 6 times with B5A medium (50 mL for each wash) and resuspended at a density of 10% (w/v), and 1 mL aliquots of the resulting suspension were plated in Petri dishes (100 × 15 mm) each containing 25 mL of the selection medium. The selection medium contained ingredients of B5A, 0.8% (w/v) Difco agar, and 10^{-7} M imazapyr (AC252,925) or imazaquin (AC252,214). Petri dishes were incubated in the dark for 2 weeks at 25°C and surviving putative resistant cell colonies were isolated and subcultured on a fresh medium of the same composition. Cell suspension cultures from the putative resistant calli were prepared by first transferring the calli (about 2 mm diameter) to 25 mL of B5A liquid medium for a week, and later, the whole of the resulting suspensions to 250 mL flasks containing 25 mL of B5A medium for another week. Thereafter, cell suspensions were subcultured normally as described above.

Growth Study of Resistant Variants

Cells were subcultured five times in B5A medium in the absence of selective herbicide prior to performing the growth assay. Growth rates were determined by a liquid growth assay. For performing a liquid growth assay, the cells were collected using a Nalgene filtration unit over Miracloth filter discs under a suction pressure of 80 K Pag (60 s) provided by an aspirator. Using a sterile spoonula, 0.5 g of cells was transferred to each flask containing the test medium, and the flasks were replaced on the shaker. Three replicates were prepared for each concentration of the inhibitors, imazapyr and imazaquin, and CS. Cells were collected again after 2 weeks and weighed to determine the growth. Increase in wet weight of cells in the absence of herbicides served as control; cell growth in the presence of a herbicide was expressed as a percentage of the control.

Assay of Acetolactate Synthase

ALS was extracted from 4-d-old cells. The methods of enzyme extraction, assay, and protein estimation were according to Ray (15) as modified previously (17).

Chemicals

Imazapyr and imazaquin were obtained from American Cyanamid Co., Princeton, NJ and chlorsulfuron from DuPont Co., Wilmington, DE. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS

A clonal cell line was established for isolating resistant variants. Cells from a clonal cell line are ideal starting material

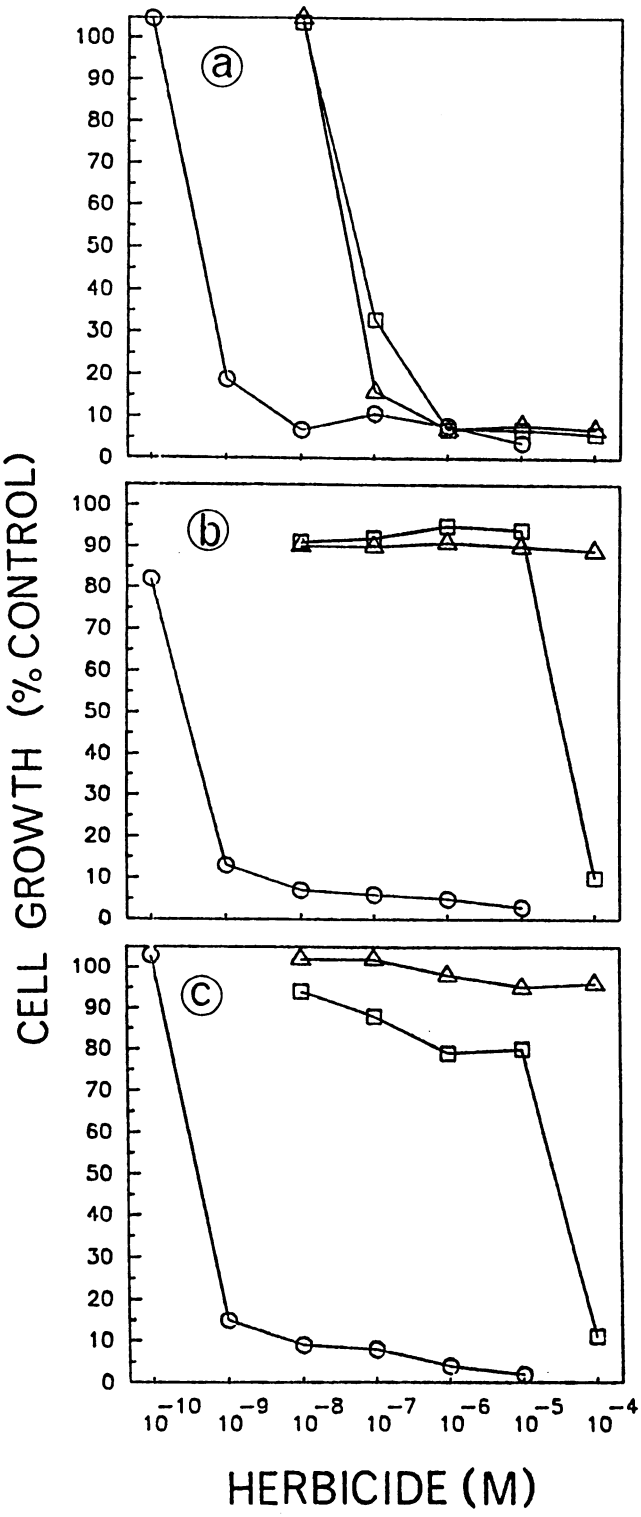

Figure 1. a–c, The effect of imazaquin (□), imazapyr (△), and chlorsulfuron (○) on the growth of wild-type and selected resistant cell lines of *D. innoxia*. The letters denote: a, wild type; b, ARR1, and c, SCR1.

for mutant/variant selection because of a relatively higher degree of physiological and genetic uniformity. Among all selected variants which showed resistance to imazapyr or imazaquin, only one cell line was chosen for further growth and enzyme analyses, thus ensuring an independent origin for each variant.

Growth of the wild-type cells was inhibited by both imazapyr and imazaquin. Of the two herbicides, imazapyr was slightly more effective than imazaquin in suppressing the growth of the wild type (Fig. 1a). However, CS caused inhibition of cell growth in wild type at much lower concentrations than either imazapyr or imazaquin. Cell growth analyses of the selected resistant variants ARR1 and SCR1 revealed a very high level of resistance (>1000-fold) to the class of herbicides against which they were selected, *i.e.* imazapyr and imazaquin, respectively (Fig. 1, b and c). The variant ARR1 was found to have cross-resistance to imazaquin; only at the 10^{-4} M level was growth severely inhibited (Fig. 1b). SCR1 cells were also highly cross-resistant to imazapyr and maintained nearly normal growth even at the highest concentration (10^{-4} M) used but were able to retain only 11% growth of the normal on the same concentration of imazaquin against which they were selected (Fig. 1c). The evaluation of cross-resistance of ARR1 and SCR1 to CS revealed that both these cell lines were sensitive to CS. Cell growth of ARR1 and SCR1 was severely suppressed at 10^{-9} M of CS and the degree of suppression was similar to that observed with wild-type cells (Fig. 1, a–c).

The activity of ALS isolated from wild-type and resistant cells was assayed in the presence and absence of imazapyr, imazaquin, and CS. The ALS from both ARR1 and SCR1 cells was highly resistant to imazapyr and imazaquin and retained >80% activity even at a 10^{-5} M concentration of the inhibitor (Fig. 2, b and c). However, ALS activity in the wild-type as well as the ARR1 and SCR1 cell lines was inhibited by CS indicating the absence of a form of ALS resistant to this herbicide in ARR1 and SCR1 (Fig. 2, a–c). The pattern of the inhibition response and herbicide dose relationship of ALS closely resembled that of growth inhibition (cf. Figs. 1 and 2).

DISCUSSION

This study with *Datura innoxia* cells demonstrates the presence of a resistant ALS in imidazolinone-resistant cell lines. The commonality of the responses of cell growth inhibition by sulfonylureas and imidazolinones and the occurrence of an altered form of ALS in sulfonylurea- and imidazolinone-resistant mutants (4–6, 9, 10, 17, 20, 23) that was insensitive to inhibition by these inhibitors supported the idea of a common site of their action. In addition to imidazolinones, another class of herbicides, the triazolo pyrimidines or sulfonanilides, has also been shown to inhibit ALS (22). In a recent investigation Schloss *et al.* (18) demonstrated the displacement of a radiolabeled sulfonylurea herbicide from bacterial ALS by imidazolinone, and triazolo pyrimidine inhibitors and by the ubiquinone homolog, Q_o. It was proposed (18) that all of these inhibitors compete for a common herbicide-specific site which is an evolutionary vestige of the

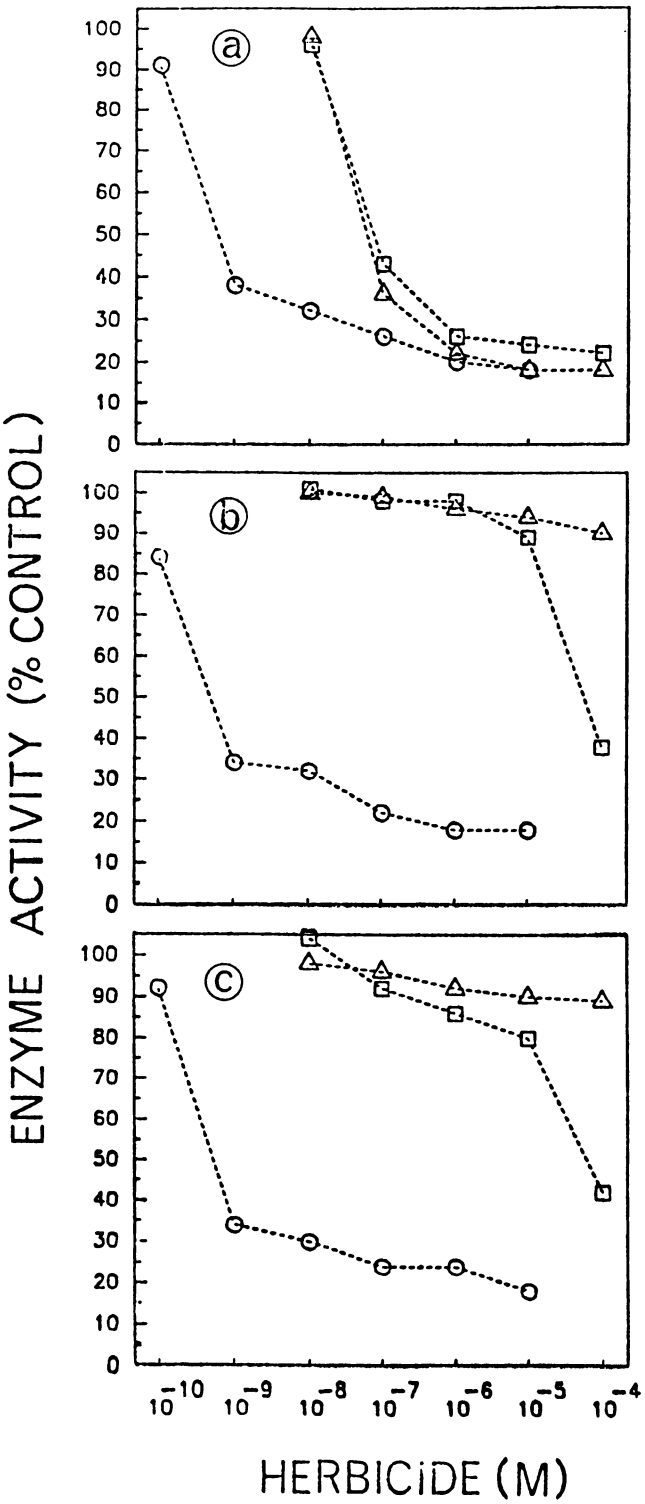

Figure 2. a–c, The inhibition of the activity of acetolactate synthase extracted from the wild-type and selected resistant cell lines of *D. innoxia* by imazaquin (□), imazapyr (△), and chlorsulfuron (○). The letters denote: a, wild type; b, ARR1, and c, SCR1.

quinone binding site of pyruvate oxidase. The theory of a common evolutionary heritage of the ALS and pyruvate oxidase emerged from the discovery of their close structural similarities (18). On the basis of such consistency in the mode of action of sulfonylureas, imidazolinones, and triazolo pyrimidine and the properties of the ALS from resistant mutants, it was reasonable to conclude that a single mutation may confer resistance to all three classes of herbicides and that the mutants resistant to one group of herbicides—sulfonylureas, for example—should naturally be resistant to the other two, *i.e.* imidazolinones and triazolo pyrimidine. Indeed, Subramanian and Gerwick (22) found that tobacco mutants resistant to triazolo pyrimidine were also cross-resistant to sulfonylureas and imidazolinones.

Interestingly, however, in our earlier study with CS-resistant variants of *D. innoxia,* we demonstrated that the cells and their ALS which showed a high level of resistance to CS were not necessarily cross-resistant to imidazolinones suggesting the possibility of separable functional sites of action on the ALS molecule (17). In a similar investigation with the CS-resistant embryogenic cell cultures of *Brassica napus,* no cross-resistance to imidazolinones was observed at the level of the cell, differentiated embryos or ALS (PK Saxena, J King, unpublished data). In a recent study (7) of transgenic CS-resistant tobacco plants recovered after genetic transfer of a mutant ALS gene, the lack of cross-resistance to imidazolinones was also noted. A logical way to determine if there were indeed separable sites of action on the ALS was to isolate mutations responsible for resistance to imidazolinones and evaluate the cross-resistance of selected cells and their ALS to CS. Thus, in this study, two cell lines of *D. innoxia,* ARR1 and SCR1, were isolated against imidazolinones which showed the presence of a form of ALS that was highly resistant to imidazolinones but sensitive to CS.

It is evident from biochemical and molecular studies that the ALS is a highly conserved gene in different organisms ranging from bacteria, algae, and yeast to higher plants. A number of previous studies performed with ALS from cultured cells and differentiated organs have shown that the ALS molecule is the site of action of a number of structurally different herbicides (3, 18). The question of the evolution of a single or separate sites of action on ALS for structurally different inhibitors is still open. Further investigation on the identification of binding site amino acids and their interaction with different classes of herbicides is needed to derive definitive conclusion on this issue. However, the existence of separable functional domains at the same binding site controlling the nature and degree of the binding of inhibitors is one plausible explanation of why several classes of compounds, such as these herbicides, can bind to and inhibit the same enzyme. Flexibility in the binding site amino acids may allow the site to accomodate a variety of approaching herbicide molecules. Particular mutations may, however, reduce this flexibility thus allowing sulfonylureas to bind, for example, but not imidazolinones, or vice versa. Varying stringency of functional domains within a single site of action may also explain the range of variation in the level of resistance and cross-resistance to structurally similar as well as distinct herbicides observed in resistant mutants. Such variation in the D1 protein found in chloroplast thylakoid membranes has been invoked as an explanation for the range of resistances some plants have been shown to display to *s*-triazine inhibitors, on the one hand, and to the structurally dissimilar inhibitor, diuron, on the other (2, 21).

The significance of the present study, together with our earlier observations (17, 24), lies in the identification of separable sites of action possibly resulting from separate mutations of the ALS gene which result in a wide range of the degree of resistance and cross-resistance to structurally similar or different herbicides. Therefore, genetic manipulation of the ALS gene so as to confer resistance to a specific herbicide or a combination of several different herbicides would seem to hold enormous potential in both fundamental and applied research. The availability of cell lines with a wide range of resistance to one or more inhibitors is expected to be very useful in elucidating the structural and functional properties of the ALS gene (14, 17).

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