Herbicide Resistance in *Datura innoxia*  

CROSS-RESISTANCE OF SULFONYLUREA-RESISTANT CELL LINES TO IMIDAZOLINONES

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**ABSTRACT**

Cells resistant to the sulfonylurea herbicides chlorosulfuron and sulometuron methyl were isolated from a predominantly haploid cell suspension culture of *Datura innoxia* P. Mill. Exponentially growing cell colonies (aggregates of about 40 cells) were mutagenized with ethyl methane sulfonate, subcultured for 10 days to allow growth recovery and plated on a medium containing either chlorosulfuron or sulometuron methyl at a concentration (10⁻⁴ molar) which killed wild type cells. Surviving clones were picked up after 3 to 4 weeks, further proliferated as callus or cell suspension cultures, and tested for their resistance to both the sulfonylureas and imidazoliones, a chemically different class of herbicides. The variants were stable and showed high (100- to 1000-fold) resistance to the sulfonylureas. While some also exhibited cross resistance to imidazoliones, others showed no cross-resistance at all or, as in one case, greater sensitivity than wild type cells to the imidazoliones. Both classes of herbicides tested inhibited acetolactate synthase activity isolated from wild type cells. The acetolactate synthase of the resistant variants, however, was found to be resistant to the sulfonylureas and also to the imidazolione(s) in those cells showing cross-resistance to the latter. The lack of cross-resistance observed in some cases provides evidence that the two groups of herbicides have slightly different sites on the acetolactate synthase molecule.

One of the expected major applications of plant biotechnological research is the engineering of herbicide resistance and its genetic transfer to crops of agronomic importance (12). The molecular understanding of the mechanisms of herbicidal action is equally important to formulate new herbicide(s) for specific applications as for many crops suitable herbicides are not known (9). For the latter purpose, it would be desirable to isolate a number of resistant mutants, independent in origin and different from each other in respect of level and also, if possible, in the mode of resistance.

We have been using predominantly haploid cell cultures of *Datura innoxia* for isolating variants resistant to the sulfonylurea class of herbicides, CS and SM, which are active ingredients of two major herbicides, Glean and Oust (DuPont), respectively. The sulfonylureas have high herbicidal activity (10–20 g·ha⁻¹) at the whole plant level and very low mammalian toxicity (3). The site of action of CS and SM in bacteria (14), yeast (5), and higher plants (2) has been shown to be the enzyme ALS (EC 4.1.3.18; also referred to as acetohydroxy acid synthase) which is the first enzyme in the biosynthesis of the branched chain amino acids, valine and isoleucine. Since the sulfonylureas inhibit cell division and growth (21), they can be used to select resistant variants *in vitro*. In higher plants there is one report of the isolation of sulfonylurea resistant variants, from tobacco cell cultures (3). Mutagenized seed populations of *Arabidopsis thaliana* have also been used for selecting CS-resistant variants (10).

Earlier we presented a preliminary report on the isolation of sulfonylurea-resistant cell lines of *D. innoxia* (22). In this communication we provide a more detailed account of the isolation and characterization of some independent variants of *D. innoxia*, resistant to CS and SM and an evaluation of the degree of their cross-resistance to two other herbicides marketed as Arsenal (AC252,925) and Scepter (AC252,214). The latter belong to a chemically different class of herbicides, the imidazoliones, which have also been shown to act by inhibiting the activity of acetolactate synthase in plants (24). Here, we demonstrate that the resistance to sulfonylureas and the cross-resistance to imidazoliones in the selected *Datura* variants arise as a result of the production of an altered acetolactate synthase which is less sensitive to those concentrations of the herbicides that inhibit the activity of the enzyme extracted from WT cells. In a variant cell line resistant to sulfonylureas but more sensitive than the WT to the imidazoliones, ALS is also shown to be the target. The ability to separate the inhibition of ALS by sulfonylurea herbicides from that by the imidazoliones suggests that there are slightly different sites on the enzyme molecule for these two groups of inhibitors.

**MATERIALS AND METHODS**

**Cell Suspension Cultures**. A predominantly haploid (>90%), WT cell suspension culture of *D. innoxia* P. Mill. obtained from an anther-driven seedling was used for the isolation of variants. Cells were cultured and maintained in BSA medium which contained ingredients of B5 medium (6) with added ammonium nitrate (500 mg·L⁻¹), 1 mg·L⁻¹ 2,4-D and 3% sucrose. The pH of the medium was adjusted to 5.6 before autoclaving at 15 p.s.i. Subculturing was carried out once a week by transferring 5 ml of cell suspension to 250 ml DeLong flasks containing 50 ml of fresh medium. These liquid cultures were maintained on a horizontal gyrahty shaker (model G-10 rotatory shaker, 2.5 cm eccentricity, New Brunswick Scientific Co., Edison, N.J.) set at 150 rpm. The stock solutions of CS and SM were prepared using acetone as solvent. Acetone was removed by taking the stock solutions to dryness under nitrogen and solutions prepared by redissolving the compounds in 5 mM potassium phosphate buffer (pH 7.5). The stock solution of Scepter (AC252,214) (2-[4,5-Dihydro-4-methyl-(1-methyl)ethyl]-5-OXO-1H-imidazole-2-Y1) - 3-quinoline carboxylic acid) was prepared by dissolving the required amount in 4% dimethylsulfoxide (2 ml) and gradually diluting it with 5 mM phosphate buffer (pH 7.5) using constant concentrations to adjust the final concentration of the cross-resistant variant.
stirring. Arsenal (AC252,925) (isopropylamine salt of 2-[4,5-di-
hydro-4-methyl-5-(1-methyl-ethyl)-5-oxo-1Himidazol-2-yl]-3-
pyridine carboxylic acid) was directly dissolved in phosphate buffer.
The highest concentrations of CS and SM used were 10^-4 M, and of
AC252,925 and AC252,214, 10^-4 and 10^-3 M, respectively. A
medium with 10^-3 M of AC252,214 could not be prepared because of
the precipitation of the compound at this concentration. All the stocks
were filter-sterilized (0.22 μm, Millipore Corp., Bedford, MA), kept at 4°C and used within a week of preparation.

Isolation of Resistant Variants. Three-d-old WT cells were passed
through a 300-μm screen (Albat Godde-Bedins Sales, Elmsford,
NY) collected over Miracloth (Chicopee Mills Inc., New York)
and resuspended in fresh culture medium (BSA). A filter-steri-
Z6lized ethyl methanesulfonate solution was added directly to the
flasks to a final concentration of 0.2 to 0.25% and the flasks
returned to a shaker for 2.5 h. Cells were then collected, washed
with BSA medium (four times, using 50 ml of the medium each
time), and subcultured in BSA medium for 10 d to allow growth
recovery. For selection, the cells mutagenized as above were
collected again over Miracloth, resuspended at a density of 10^6
(w/v) in fresh BSA medium supplemented with either 10^-5 M CS
or SM and transferred to selection dishes (1 ml/10 cm diameter
Petri dish). Each selection dish contained 25 ml BSA medium
enriched with the herbicide and solidified with 0.7% (w/v) agar.
Petri dishes were sealed with Parafilm and incubated in the dark
for 4 weeks. Growing cell colonies were picked up and further
subcultured on fresh selection medium, for obtaining callus masses.
For obtaining cell suspension cultures of resistant variants, small
pieces (2-3 mm in diameter) of the calli were transferred to 10
ml medium lacking herbicide dispensed in 125 ml flasks and kept
on a shaker (100 rpm). The medium was replenished every 5 d
by adding 3.5 ml of fresh medium. After about 20 d whole sus-
pensions were transferred to 250 ml flasks and maintained like
WT cells.

Growth Study of Resistant Variants. Growth of the variant
cells was measured by the filter paper growth assay as described
by Horsch et al. (11). Briefly, the exponentially growing cells
were collected over Miracloth, suspended in BSA medium at a
density of 20% (w/v) and continuously stirred in a 100 ml beaker.
One ml aliquots of cell suspension were gently pipetted onto
disks of 7 cm diameter filter paper (Whatman No. 2. Qualitative)
placed on agar-solidified medium in 10-cm diameter Petri dishes.
The dishes were gently tilted and rotated to distribute the cells,
and weighed under sterile conditions. The filter papers were
washed with the dishes from sterile forceps, the dishes weighed
again, and the filter papers replaced. At 2-d intervals for 2 weeks,
the dishes and filter papers with inocula were reweighed in the
same way, the difference giving the wet weight of the growing
cultures. The increase in cell weight was expressed as percentage
of control, i.e. weight of cells accumulated in the absence of the
herbicide.

Assay of Acetolactate Synthase. ALS was extracted from 2-d-
old cell suspension cultures using methods of enzyme extraction
and assay modified from those of Challef and Mauvais (2) and
Ray (20). Cells were collected over Miracloth by suction and
washed twice with 100 ml of glass-distilled water, collected again,
and suspended in two volumes of a buffer containing 0.1 M po-
tassium phosphate (monodibasic, pH 7.5), 1 mM sodium pyru-
vate, 0.5 mM MgCl₂, 0.5 mM thiamine pyrophosphate, 10 mM
flavin adenine dinucleotide, 10% (v/v) glycerol, and polyvinyl-
polypyrrolidone (1.25 g/6 g cells). Glass beads (2 mm) were
added to the cells at a 3:1 (beads:cells; w/w) ratio and the sus-
pension homogenized in a Braun homogenizer (type 2876) cooled
with CO₂. Glass beads were removed by filtration through a 500-
μm screen and the cell debris by centrifugation at 2500g. The
25 to 50% ammonium sulphate fraction was precipitated from
the supernatant, redissolved in elution buffer (0.1 M potassium
phosphate, pH 7.5) containing 20 mM sodium pyruvate and 0.5
mM MgCl₂ and diluted to the required volume.

The assay for acetolactate synthase was carried out at 30°C for
45 min in a 0.5 ml final volume of the reaction mixture containing
20 mM phosphate buffer (pH 7.0), 20 mM sodium pyruvate, 0.5
mM thiamine pyrophosphate, 0.5 mM MgCl₂, 10 μM flavin adi-
enine dinucleotide, different concentrations of herbicides, and
100 μl of the extracted enzyme. The conversion of acetolactate
to acetoacetin by H₂SO₄ and NaOH and the determination of acetoacetin
was carried out at 65°C according to the procedure of Westerfield
(26) as modified by Ray (20). Protein concentrations were
determined with the Bio-Rad protein assay.

Chemicals. Chlorsulfuron and Sulfometuron methyl were ob-
tained from DuPont Co., Wilmington, DE, and Arsenal and
Scepter from the American Cyanamid Co., Princeton, N.J. All
other chemicals were purchased from Sigma Chemical Co., St.
Louis, MO.

RESULTS

Effect of Herbicides on Cell Growth. The growth of WT cells
at various concentrations of the herbicides is shown in Figure
1. The CS and SM were completely inhibited WT cells.
Selection was carried out at 10^-6 M concentrations of these her-
bitides, well above the concentration shown to inhibit the growth
of WT cells. The WT cells were much less sensitive to the imi-
dazolones than to the sulfonylureas. Only at a concentration of
10^-5 M was growth completely inhibited by AC252,925 and
AC252,214 (Fig. 1a).

Mutagenized WT cells were plated at a density of about
8 x 10^5 cells per plate made up of about 2 x 10^6 colony forming
units each containing about 40 cells. Clones which survived 10^-8
M of either CS or SM were transferred at 3 to 4 weeks to a
medium containing 5 x 10^-8 M of the same herbicide. Those
clones capable of sustained growth at this concentration were
considered to be putative resistant variants and were analyzed
further.

Figure 2 shows the growth of WT and a representative resistant
callus on medium with and without CS. Twenty-one such variants
were isolated in five separate experiments with a frequency of 1
to 3 variants per 10^6 mutagenized cells. Of these, six clones of
independent origin were studied in further detail.

The variants CSR1, CSR2, CSR6, CSR10, and CSR18 were
isolated using CS; SMR1 with SM. They showed wide variation
in their resistance to the sulfonylureas and cross-resistance to the
imidazolones when challenged again with the herbicides after
three to four passages in nonselective medium. SMR1 was only
moderately (10- to 15-fold) resistant to CS, SM, and AC252,214
but highly resistant to AC252,925 (1000-fold) with an IC₅₀ at 10^-5
M as compared to 3.10^-8 M of the WT (Table I). The variants
CSR1 (Fig. 1b), CSR6 and CSR10 (Table I) were all moderately
(100-fold) to highly (1000-fold) resistant to both the sulfonylurea
and imidazolone herbicides. In contrast, CSR18 although highly
resistant to the sulfonylureas was not at all resistant to the imi-
dazolones (Fig. 1c). The variant, CSR2, differed from all others in
being more sensitive than WT cells to both imidazolines-
IC₅₀ at 3.10^-10 and 10^-8 M for AC252,214 and AC252,925,
respectively, while showing high resistance to the sulfonylureas
(Table I).

Effect of Herbicides on Acetolactate Synthase. ALS has been
shown to be the site of action of both sulfonylurea and imida-
zolone herbicides in plants (2, 24). In the case of ALS from
WT Datura cells, 50% inhibition of enzyme activity occurred at
10^-3 M for both CS and SM compared to 10^-2 M for AC252,214
and 10^-5 M for AC252,925 (Fig. 3a). The patterns of enzyme
inhibition in the case of the variants with (CSR1, Fig. 3b) or
without cross-resistance (CSR18, Fig. 3c) to imidazolines were
could be cloned and used. Datura
haploid are known to have an independent origin. Calli of Datura (E-), CS, and WT and variant calli (100-fold) were tested. The letters denote: a, WT; b, CSR1; c, CSR18. Each point in the figure is an average of six replicates from two different experiments. The standard error for any point was less than 10%.

All variants have remained stable, on and off pressure, since their isolation over a year ago during which time cells have undergone more than 250 divisions. Attempts to regenerate plants from calli of the resistant variants have so far been unsuccessful. The WT and variant calli turn green and produce nodular, hairy callus with protuberances resembling shoot-bud primordia on MS (18) medium containing benzyladenine or zeatin (2–10 μM).

**DISCUSSION**

The present study was undertaken for the purpose of isolating from haploid Datura cell cultures a wide range of variants of independent origin resistant to the sulfonylurea herbicides which are known to have a specific metabolic target. It was hoped to generate material from which the genes conferring resistance could be cloned and used for genetic transformation. Haploid Datura cell cultures were chosen because they have been used successfully to generate auxotrophic variants in this laboratory (13) and because they are very sensitive (about 3 × 10⁻¹⁰ M) to the sulfonylurea herbicides.

Although CS and SM were equally lethal to cell growth, variants with a high degree of resistance were recovered only when CS was the selection agent. SMR1 (see “Results”) and seven other SMR-type variants (data not shown) were less resistant to CS and SM than CSR-type clones. The reason why is not clear since SM has been used effectively to generate resistant mutants in Saccharomyces cerevisiae (5).

Sulfonylurea herbicides block plant growth by inhibiting the activity of ALS, the first enzyme in the pathway of branched-chain amino acid biosynthesis (2). Resistance to the sulfonylurea herbicides has been shown to be the result of an ALS which is less sensitive to these herbicides (2). Our results confirm that resistance to the sulfonylureas is conferred by a modified ALS.

Imidazolinones also appear to inhibit plant growth by affecting ALS (24). In view of these reports, we tested the Datura variants for cross-resistance to these herbicides and found a widely variable response. SMR1, CSR1, and CSR6 were highly resistant to one or both of AC252,214 and AC252,925, responses which were again attributable to altered ALS. CSR10 was only weakly resistant to the imidazolinones and CSR18 showed no resistance. CSR2 was highly resistant (>100-fold) to the sulfonylureas but significantly more sensitive (about 100-fold) than WT cells to the imidazolinones. Interestingly, SMR1, which was not very resistant to CS or SM, showed a high degree (1000-fold) of resistance to AC252,925. Again, ALS modification seemed to be the most probable source of the variability. It is therefore apparent that the production of a resistant ALS is one way to acquire resistance to the imidazolinones.

These observations support the earlier findings of Anderson and Georgson (1) and Shaner et al. (25) who isolated maize mutants resistant to imidazolinones and showed that the resistance resulted from mutational alterations of the ALS molecule. The inhibition of cell divisions leading to a block in growth by sulfonylureas and imidazolinones and the reversal of inhibition in the presence of isoleucine and valine in both cases (20, 24) indicate the commonality of their site of action. The idea of a common site of action for sulfonylureas and imidazolinones is further supported by the correlation between the acquired resist-
Table I. Concentrations of CS, SM, AC252,214, and AC252,925 Required to Inhibit Cell Growth (C) and the Activity of ALS (A) by 50% \( (I_{50}) \)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CS</th>
<th>SM</th>
<th>AC252,215</th>
<th>AC252,925</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>WT</td>
<td>(10^{-10})</td>
<td>(10^{-9})</td>
<td>(5 \cdot 10^{-11})</td>
<td>(10^{-9})</td>
</tr>
<tr>
<td>SMR1</td>
<td>(3 \cdot 10^{-9})</td>
<td>(10^{-7})</td>
<td>(3 \cdot 10^{-9})</td>
<td>(10^{-7})</td>
</tr>
<tr>
<td>CSR2</td>
<td>(3 \cdot 10^{-8})</td>
<td>(10^{-5})</td>
<td>(3 \cdot 10^{-8})</td>
<td>(10^{-5})</td>
</tr>
<tr>
<td>CSR6</td>
<td>(3 \cdot 10^{-7})</td>
<td>(10^{-4})</td>
<td>(3 \cdot 10^{-7})</td>
<td>(10^{-4})</td>
</tr>
<tr>
<td>CSR10</td>
<td>(3 \cdot 10^{-8})</td>
<td>(10^{-5})</td>
<td>(10^{-8})</td>
<td>(10^{-5})</td>
</tr>
</tbody>
</table>

* 50% inhibition was not achieved even at the highest concentration used.

Fig. 3. Activity of acetolactate synthase from the WT and the resistant variants in the presence of CS (---), SM (-----), AC252,925 (-- ---), and AC252,214 (--- - -). The letters denote: a, WT; b, CSR1; c, CSR18. Each point in the figure is an average of six replicates from two different experiments. The standard error for any point was less than 5%.

A resistant to both groups of herbicides and the production of a resistant ALS. It is therefore logical to assume that the cells resistant to sulfonylureas should also have cross-resistance to the imidazolinones and vice versa. In this context, the most noteworthy observation of the present study is the lack of cross-resistance in some of the sulfonylurea resistant Datura variants to the imidazolinones, suggesting the possibility of two slightly different sites of action. This is the first evidence to show that the sulfonylureas and the imidazolinones may have two different sites of action on the ALS molecule.

The observation of differential sensitivity of variants to the herbicides suggests the possibility of mutations having occurred at different genetic loci. Falco and Dumas (5) showed in S. cerevisiae that mutations at three separate loci each conferred resistance but to different degrees. With cultured plant cells the possibility of generating varying levels of resistance to one particular herbicide or to several herbicides of different chemical composition more than one way is strong, especially since considerable genetic variability is known to occur during the process of continuous culture and rapid cell divisions (4).

Interestingly, in the present study one of the sulfonylurea-resistant variant (CSR2) showed greater sensitivity to imidazolinones than the WT. No such sulfonylurea resistant variant of cell culture origin has been reported previously. However, several cases of enhanced sensitivity of weed biotypes—resistant to a particular PSII herbicide—to other herbicides have been reported (7, 15, 16). For example, atrazine resistant weed biotypes were killed by diuron which belongs to the same class of chemical compounds (8) and is believed to have the same binding site as atrazine on the basis of binding competition studies (7, 15). Similarly, a diuron-resistant variant of the alga Chlamydomonas was found to be sensitive to several other PSII herbicides, e.g., phenoxoles, phenylurea, and atrazine, despite the structure-activity complementarity and other biochemical evidence supporting a common binding target area for these herbicides (7). Lawrence et al. (15) proposed that when a herbicide binds to a site it causes a conformational change that may be propagated to binding sites for other herbicides modifying their affinity. The exposure of the other interacting binding sites can explain why plants (or cells) with resistance to one particular herbicide are sensitive to others. However, the possibility of a single mutation conferring resistance to more than one related or unrelated inhibitors also exists. For example, in S. cerevisiae, Rank et al. (19) have described mutants with pleiotropic drug resistance which are resistant to herbicides because they have plasma membranes with reduced permeability. No such variants have yet been isolated from Da- tura or any other cell cultures although CSR6, during the first few weeks after its isolation, showed resistance by having a presumptive, transient, tandem duplication of the ALS gene (27). Recently, more than 20 additional variants have been isolated using CS as the selection agent and are being screened for resistance by means other than an altered ALS.

The precise mode of action of sulfonylurea and imidazolinone herbicides is not yet known. Enzyme kinetic studies of ALS from Salmonella typhimurium (14) and from maize cell suspension...
cultures (17) indicate that SM and AR, respectively, bind to the enzyme slowly but tightly. The different degrees of resistance among these Datura variants and the differential sensitivity to herbicides of the ALS enzyme in each case could facilitate attempts to understand the structural-functional properties of this enzyme and the gene responsible for its production. Further, variants like CSR6 showing apparent gene amplification (27) could be useful as vehicles for cloning the ALS gene. Combining different resistant phenotypes by protoplast fusion should allow us to evaluate the possibility of engineering clones with a high degree of resistance to particular herbicides. The importance of synthesis of a somatic hybrid by protoplast fusion between two variants with resistance to two or more different herbicides has been suggested (12) but has never been tested presumably because of unavailability of such variants. Viable protoplasts can be produced in large numbers from all variants reported here and are being used to test this possibility.

All variants were stable in culture over many generations. Unfortunately, it was not possible to produce plants from either WT or variant cells although some regenerative capacity was found in all cases. Datura cultures may differ widely in their ability to regenerate (23). The genetic analysis of regenerated sulfonylurea-resistant tobacco plants showed that resistance is inherited as a dominant or semidominant mutation (3).

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LITERATURE CITED


2. CHALEFF RS, CJ MAUVAIS 1984 Acetolactate synthase is the site of action of two sulfonylurea herbicides in plants. Science 224: 1443–1445


10. HAUGHN GW, CS SOMERVILLE 1986 Sulfonylurea resistant mutants of Arabidopsis thaliana. Mol Gen Genet 204: 430–434


17. MUIHITCH JM, DL SHANER, MA STIDHAM 1987 Imidazoliones and acetohydroxyside synthase from higher plants—properties of the enzyme from maize suspension culture cells and evidence for the binding of imazapyr to acetohydroxyside synthase in vivo. Plant Physiol 83: 451–456


22. SAXENA PK, J KING 1986 Isolation of Datura innoxia cell lines resistant to sulfonylurea herbicides. In DA Somers, BG Gengenbach, DD Biesboer, WP Hackett, CE Green, eds, VI International Congress of Plant Tissue and Cell Culture (Abstracts). University of Minnesota, Minneapolis, p 96


