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Site of Action of Chlorsulfuron

INHIBITION OF VALINE AND ISOLEUCINE BIOSYNTHESIS IN PLANTS

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ABSTRACT

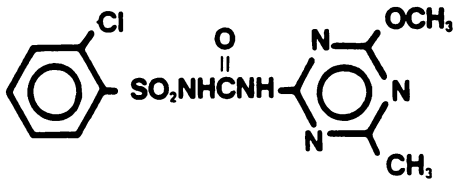
The sulfonylurea herbicide chlorsulfuron blocks the biosynthesis of the amino acids valine and isoleucine in plants. Addition of these two amino acids to excised pea root (*Pisum sativum* L. var Alaska) cultures incubated in the presence of chlorsulfuron completely alleviates herbicide-induced growth inhibition. The site of action of chlorsulfuron is the enzyme acetolactate synthase which catalyzes the first step in the biosynthesis of valine and isoleucine. This enzyme is extremely sensitive to inhibition by chlorsulfuron having I_{50} values ranging from 18 to 36 nanomolar. In addition, acetolactate synthase from a wide variety of tolerant and sensitive plants species is highly sensitive to inhibition by chlorsulfuron.

The selective sulfonylurea herbicide, chlorsulfuron (Fig. 1), represents a major advancement in weed control technology combining high herbicidal activity with outstanding tolerance to cereal crops and very low mammalian toxicity (3). Sensitive weeds are controlled at 10 to 20 g/ha which is equivalent to 1 to 2 tablespoons of active ingredient per acre. Chlorsulfuron acts to inhibit plant growth by blocking some process necessary for plant cell division (7–9). Recent studies have shown that another sulfonylurea herbicide, sulfometuron methyl, which is the active ingredient in Oust® Weed Killer (Fig. 1: Ref. 6), interferes with the production of branched-chain amino acids in bacteria by inhibiting the enzyme acetolactate synthase (2). This enzyme catalyzes the first step in the biosynthesis of Val, Leu, and Ile. Results reported here demonstrate that in plants chlorsulfuron and sulfometuron methyl also block the biosynthesis of Val and Ile by inhibiting the enzyme acetolactate synthase. These herbicides are extremely potent inhibitors of acetolactate synthase from plants having I_{50} values as low as 18.5 nM for chlorsulfuron and 15.4 nM for sulfometuron methyl.

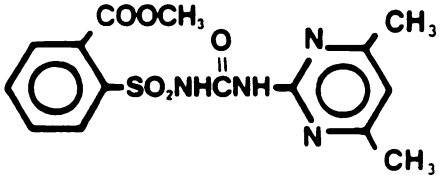
MATERIALS AND METHODS

Pea Root Culture. Peas (*Pisum sativum* L. var Alaska) were surface sterilized by two 3-min soakings with gentle stirring in commercial bleach (5.25% NaOCl). After surface sterilization, the peas were sown in moist sterile vermiculite and incubated under sterile conditions for 72 h at 25 °C. Root sections, 10 mm long, including the root tip were excised from the resulting seedlings and grown in White's medium (12) supplemented with 20 g/l of sucrose as described by Van't Hof (10). All manipulations were carried out under sterile conditions. The pH of the culture medium was adjusted to 6.5 prior to autoclaving. Stock solutions of chlorsulfuron were prepared to a concentration of 500 mg/l in tetrahydrofuran. Aliquots of this stock were taken to dryness under nitrogen in small vials and the chlorsulfuron was then dissolved in 5 mM K_2HPO_4 (pH 7.5), diluted with phosphate buffer, and filter sterilized. These precautions were taken to avoid introducing organic solvents into the root cultures. Filter sterilized solutions of chlorsulfuron, casein hydrolysate (acid hydrolyzed), and L-amino acids were added to freshly inoculated sterile cultures of pea roots under sterile conditions. Culture inoculum consisted of 10 root sections which were added to 50 ml of White's medium in 250 ml Erlenmeyer flasks (12). The roots were grown in the dark at 25°C with gentle gyrotory shaking (60 rpm) and were collected after 96 h of incubation. Growth was determined by measuring root length. The net growth of the roots was determined by subtracting the initial 10 mm from the final length.

Whole Plant Culture. Pea seeds were surface sterilized by two 3-min rinses in full strength commercial bleach (5.25% NaOCl) followed by rinsing with 1 L of sterile distilled H_2O. The surface-sterilized peas were placed on sterile Linsmaier and Skoog medium (4) containing 1.6% potato dextrose agar but omitting plant growth hormones. Solutions of chlorsulfuron and L-amino acids were prepared as described above and added to autoclaved medium. All steps were performed under sterile conditions. The



Chlorsulfuron



Sulfometuron Methyl

FIG. 1. The chemical structures of chlorsulfuron and sulfometuron methyl.

seeds were germinated and the resulting plants were grown under constant light at 25°C.

Acetolactate Synthase Extraction and Assays. Acetolactate synthase was extracted from 7- to 8- d-old etiolated pea shoots which were grown under nonsterile conditions in vermiculite at 25°C. The shoots were homogenized in 2 volumes of buffer containing 0.1 M K_2HPO_4, pH 7.5, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine-pyrophosphate, 10 μM FAD[1] and 10% v/v glycerol. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 27,000g for 20 min. Acetolactate synthase was precipitated from the supernatant fluid with $(NH_4)_2SO_4$. The enzyme was collected at 25 to 50% saturation by centrifugation and the pellet dissolved in buffer containing 0.1 M K_2HPO_4, pH 7.5, 20 mM pyruvate, and 0.5 mM $MgCl_2$ and desalted on small column of Sephadex G-25 (Pharmacia PD-10) equilibrated with the same buffer. The desalted enzyme was used immediately for assays.

Acetolactate synthase assays were carried out in a final volume of 0.5 ml at 30°C. The final reaction mixture contained 20 mM K_2HPO_4 pH 7.0, 20 mM sodium pyruvate, 0.5 mM thiamine-pyrophosphate, 0.5 mM $MgCl_2$, 10 μM FAD, and various concentrations of chlorsulfuron. Chlorsulfuron was prepared as described above to avoid the introduction of organic solvents into the reaction mix. Assays were initiated by adding enzyme (100 μl) and terminated by adding 50 μl of 6 N H_2SO_4. Acetolactate was determined as described by Westerfield (11) with the following modifications. The acidified reaction mixtures were heated for 15 min at 60°C after which 0.5 ml of 0.5% w/v creatine was added. Next, 0.5 ml of 5% w/v α-naphthol, freshly prepared in 2.5 N NaOH, was added and the solutions were heated for an additional 15 min at 60°C. The absorbances of the solutions were then determined at 525 nm. Protein was determined by the method of Lowry et al. (5).

The I_{50} value for inhibition is defined as the concentration of chlorsulfron which inhibits acetolactate synthase activity 50% in a 30-min fixed time assay carried out as described above. The following equation was used to calculate the I_{50}:

$$\% \text{ Activity} = 100/(1 + [SU]/I_{50})$$

where % activity equals the amount of activity in the presence of various sulfonylurea concentrations as per cent of an untreated control, and SU equals the sulfonylurea concentration. The I_{50} was calculated by a least-squares method using the RS/1 Fit Function Curve Fitting Program (BBN Research Systems Inc.). Values are the average of at least two determinations. The standard deviations of regression for the fits were between 2 to 3%.

RESULTS

Excised pea roots grown in sterile culture provided a quantitative and sensitive bioassay for evaluating the effects of chlorsulfuron on plant growth. As little as 2.8 nM of the herbicide significantly inhibited root growth while 28 nM typically inhibited root growth by more than 80% (Fig. 2). Casein hydrolysate at 0.1% w/v in the culture medium, however, provided a significant reversal of this growth inhibition throughout the entire range of chlorsulfuron concentrations tested (Fig. 2). Thus, chlorsulfuron-induced growth inhibition in plants can be reversed at least in part by casein hydrolysate as has been reported for bacteria (2).

To determine if an amino acid or group of amino acids in the casein hydrolysate was responsible for the observed protection, 20 L-amino acids were added to the root cultures in groups based on their common biosynthetic pathways (Table I). The only group of amino acids which alleviated chlorsulfuron-induced

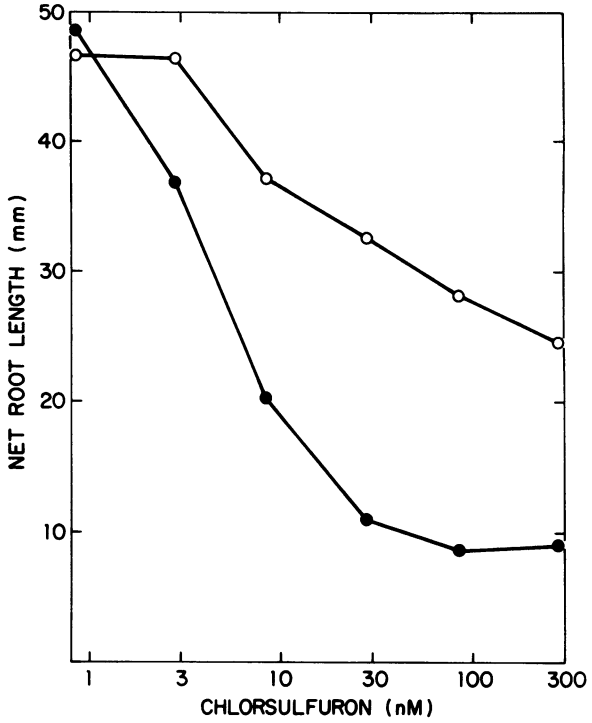


FIG. 2. The effect of casein hydrolysate on the growth of pea roots in the presence of various concentrations of chlorsulfuron. Each point is the average net length of 10 roots. Control without casein hydrolysate supplement (●); with 0.1% w/v casein hydrolysate supplement (○).

growth inhibition contained 100 μM each of Val, Leu, Ile, and Ala. Subsequent tests revealed that additions of Val plus Ile alone were effective in providing protection (Table II). Neither Leu, Ile, nor Ala alone nor in combination with each other could alleviate the growth inhibition. Val alone was partially effective in reversing chlorsulfuron-induced growth inhibition.

When tested against various concentrations of chlorsulfuron, a combination of 100 μM each of Val and Ile completely protected the roots from growth inhibition in the presence of up to 280 nM chlorsulfuron (Fig. 3). Thus, the presence of these two amino acids can reverse the inhibitory effects of concentrations of chlorsulfuron which are at least 100 times that needed to inhibit growth.

The protective effects of casein hydrolysate and Val and Ile could not be explained on the basis of a general stimulation of growth since the controls with or without the supplements were not significantly different (Tables I and II).

The ability of Val and Ile to reverse chlorsulfuron-induced growth inhibition can also be demonstrated in whole plants. Nearly complete growth inhibition is apparent when peas are germinated in the presence of 28 nM chlorsulfuron (Fig. 4). However, when 100 μM each of Val and Ile are included in the growth medium along with the chlorsulfuron, normal growth occurs.

These results strongly suggested that chlorsulfuron interferes with the production of Val and Ile. Accordingly, the site of action of chlorsulfuron was further defined by supplementing pea root cultures with intermediates of the Val-Ile biosynthetic pathway. In the presence of 28 nM chlorsulfuron, 100 μM pyruvate and threonine, the initial intermediates in the Val-Ile pathway, did not alleviate growth inhibition. However the penultimate intermediates, α-ketoisovaleric acid and α-keto-β-methylvaleric acid, although somewhat inhibitory to root growth by themselves, provided significant protection (Table III). The lack of complete protection with these two ketoacids may have been due to their

[1] Abbreviations: FAD, flavin adenine dinucleotide.

Table I. *Effect of Amino Acid Groups on Chlorsulfuron Inhibition of the Growth of Excised Pea Roots*

The final concentration of each amino acid was 100 μM. The values are given as the average net length of 10 roots ± the SE.

Amino Acid Group	Avg. Net Root Length	
	Control	28 nM Chlorsulfuron
	mm	
No Addition	35.1 ± 1.8	10.1 ± 0.9
Ser Gly Cys	31.7 ± 1.2	9.3 ± 0.9
Glu Gln Arg	38.6 ± 1.4	8.6 ± 0.5
Ala Leu Ile Val	32.2 ± 1.3	32.3 ± 2.9
Phe Tyr Trp	27.2 ± 2.1	8.8 ± 0.6
Asp Asn Lys Met Thr	32.4 ± 3.7	7.7 ± 0.9
His	32.1 ± 2.5	9.6 ± 1.3

Table II. *Effect of Various Combinations of Val, Leu, Ile, and Ala on the Growth of Excised Pea Roots*

The concentration of each amino acid was 100 μM. The values are the average net lengths of 10 roots ± the SE.

Supplement	Avg. Net Root Length	
	Control	28 nM Chlorsulfuron
	mm	
No Addition	52.8 ± 1.1	10.3 ± 0.3
Leu, Ile, Ala, Val	48.8 ± 1.8	50.2 ± 1.0
Leu, Ala, Val	49.2 ± 1.6	29.3 ± 0.5
Leu, Ile, Ala	44.1 ± 1.2	11.1 ± 0.5
Leu, Ile, Val	42.9 ± 1.0	47.4 ± 0.8
Ile, Ala, Val	45.0 ± 1.1	34.1 ± 1.2
Leu, Ile	24.7 ± 2.3	11.0 ± 0.5
Leu, Val	50.9 ± 1.0	18.8 ± 0.5
Leu, Ala	50.9 ± 1.0	10.7 ± 0.3
Ile, Val	44.8 ± 0.8	42.0 ± 1.2
Ile, Ala	50.9 ± 0.9	10.7 ± 0.4
Val, Ala	44.6 ± 1.1	20.2 ± 1.3
Leu	26.1 ± 2.4	11.9 ± 0.5
Ala	54.1 ± 1.5	10.4 ± 0.5
Ile	34.8 ± 0.8	10.1 ± 0.3
Val	47.6 ± 1.1	20.9 ± 0.5

lability or only partial uptake by the roots.

Collectively, the results of the supplementation experiments described above indicated that chlorsulfuron acts on one of the primary steps of the Val-Ile pathway. One characteristic of this pathway is that a common set of enzymes catalyze the biosynthesis of both amino acids. Acetolactate synthase (EC 4.1.3.18),

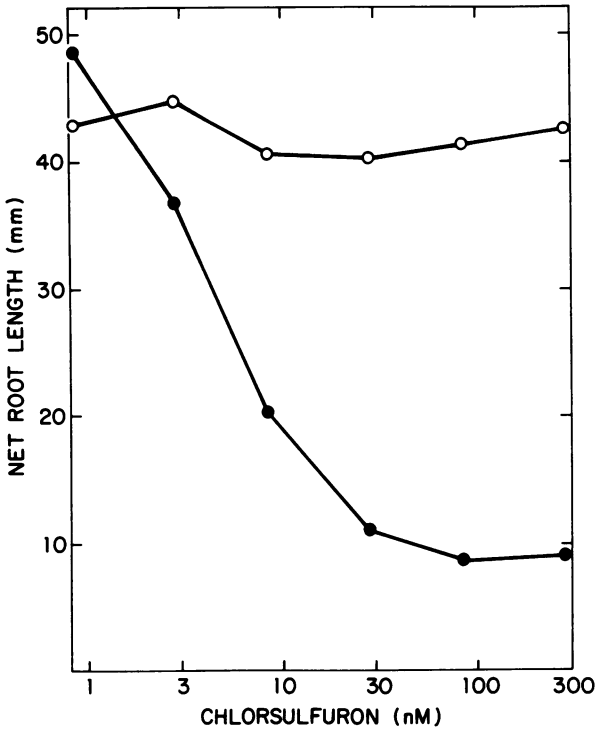


FIG. 3. The effect of 100 μM each of Val and Ile on the growth of pea roots in the presence of various concentrations of chlorsulfuron. Each point is the average net length of 10 roots. Control without amino acid supplement (●); with 100 μM valine and isoleucine (○).

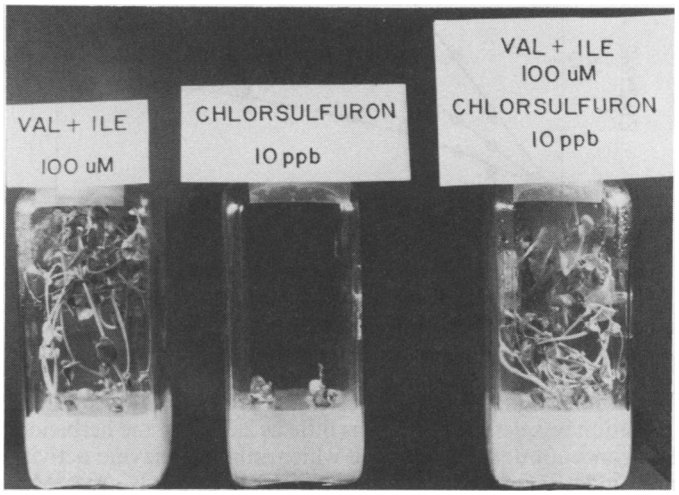


FIG. 4. The effects of Val and Ile on the growth of pea seedlings in the presence of 28 nM (10 ppb) chlorsulfuron.

the first common enzyme in the Val-Ile pathway was extracted from pea shoots and tested for sensitivity to inhibition by chlorsulfuron. The enzyme was found to be strongly inhibited by the herbicide with significant inhibition of product formation evident at 28 and 84 nM chlorsulfuron (Fig. 5) The progress curves for acetolactate synthase inhibition by chlorsulfuron indicate that the amount of inhibition increases with time when the enzyme is assayed in the continued presence of the herbicide with the rate of inhibition a function of the inhibitor concentration (Fig. 5). Similar kinetics have been reported for sulfonylurea inhibition of acetolactate synthase II from *Salmonella typhimurium* (2).

A dose response curve for the inhibition of acetolactate synthase is shown in Figure 6. The data were obtained from 30-min

Table III. *Effect of Various Intermediates of the Val-Ile Pathway on the Growth of Excised Pea Roots in the Presence of 28 nM Chlorsulfuron*

The values are the average net lengths of 10 roots ± the SE.

Supplement	Concn.	Avg. Net Root Length	
		Control	28 nM Chlorsulfuron
	mM	*mm*	
No additions		49.6 ± 0.8	5.8 ± 0.4
Pyruvate + threonine	0.1	51.0 ± 1.3	6.5 ± 0.5
α-Ketoisovalerate + α-keto-β-methylvalerate	1	29.8 ± 1.0	20.6 ± 0.6
Valine + isoleucine	0.1	44.8 ± 1.4	41.6 ± 1.2

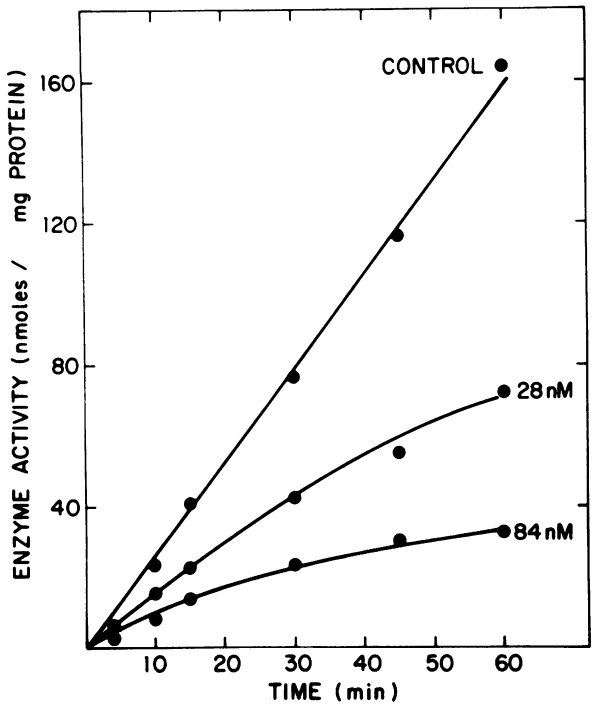


FIG. 5. Rate curves for acetolactate formation by acetolactate synthase in the presence of 28 nM and 84 nM chlorsulfuron. The acetolactate formed was measured as acetoin as described in "Materials and Methods".

fixed time assays in the presence of up to 84 nM chlorsulfuron. Inhibition was detectable with as little as 2.8 nM of the herbicide. The concentration of herbicide which inhibits enzyme activity 50% under these assay conditions (defined as the I_{50}, see "Materials and Methods") is 21 nM. Sulfometuron methyl also was found to be a potent inhibitor of acetolactate synthase with an I_{50} of 15.4 nM.

Acetolactate synthase from plants other than peas is also sensitive to inhibition by chlorsulfuron. Table IV lists a number of species from which acetolactate was extracted and then assayed in the presence of chlorsulfuron. Included in this list are both broadleaves and grasses. In all cases the acetolactate synthase activity is sensitive to inhibition with the I_{50} values all ranging between 17 and 34 nM for chlorsulfuron.

DISCUSSION

The results presented here indicate that the sulfonylurea herbicides, chlorsulfuron and sulfometuron methyl, act on plant tissue by blocking the biosynthesis of the amino acids Val and Ile. This block results from the herbicides directly inhibiting the

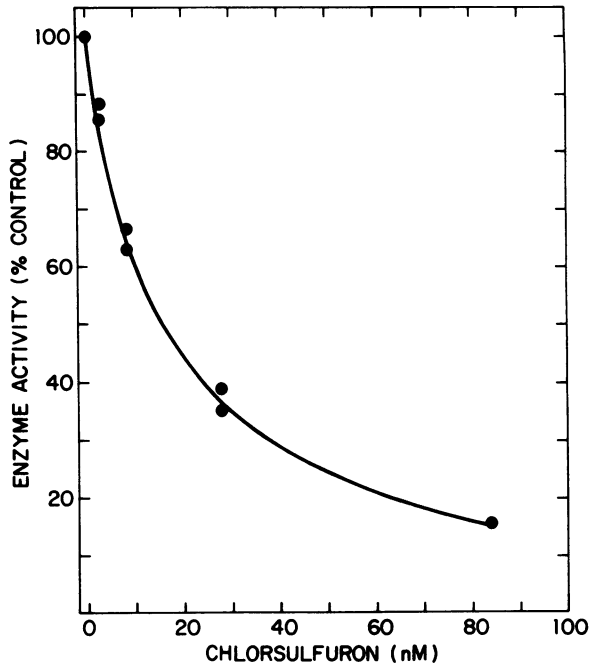


FIG. 6. Dose response for the inhibition of acetolactate synthase by chlorsulfuron. The values are given as the per cent of an untreated control. The specific activity of the control was 220 nmol/h·mg protein. The acetolactate formed was measured as acetoin as described in "Materials and Methods".

Table IV. *I_{50} Values for Chlorsulfuron Inhibition of Acetolactate Synthase from Various Plant Species*

Species	I_{50}
	nM
Pea	21.0
Wheat	18.5
Soybean	23.0
Tobacco	28.3
Green foxtail	25.8
Johnsongrass	35.9
Morning glory	24.4

enzyme acetolactate synthase which catalyzes the first step in the biosynthetic pathway for these two amino acids. The concentrations needed to inhibit acetolactate synthase are within the range necessary to inhibit plant growth as determined with the pea root cultures. Inhibition of enzyme activity increases with time in the continued presence of chlorsulfuron (Fig. 5). Thus, the enzyme may be even more sensitive to inhibition than indicated here, since even very low concentrations of chlorsulfuron would eventually cause inhibition.

In addition to biochemical evidence presented here, there are also substantial genetic data which confirm that acetolactate synthase is the site of action of sulfonylurea herbicides in plants. Recently it has been demonstrated that tobacco plants containing a single dominant nuclear gene mutation which confers resistance to chlorsulfuron have an altered form of acetolactate synthase which is far less sensitive to inhibition by this herbicide (1; R. Chaleff and C. J. Mauvais, unpublished data). Through a series of crosses, it was demonstrated that the chlorsulfuron insensitive acetolactate synthase cosegregates with the chlorsulfuron-resistant phenotype. Taken together, the biochemical and genetic data provide strong evidence that acetolactate synthase is the primary site of action of sulfonylurea herbicides such as chlorsulfuron and sulfometuron methyl in plants.

Previous studies on the mechanism of action of chlorsulfuron

have demonstrated that this herbicide rapidly inhibits plant cell division (7). Results presented here show that the basis for this effect is the inhibition of the biosynthesis of the essential amino acids Val and Ile.

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LITERATURE CITED

1. CHALEFF RS, TB RAY 1984 Herbicide-resistant mutants from tobacco cell cultures. Science 223: 1148–1151
2. LAROSSA RA, JV SCHLOSS 1984 The herbicide sulfometuron methyl is bacteriostatic due to inhibition of acetolactate synthase. J Biol Chem. In press
3. LEVITT G, HL PLOEG, RC WEIGEL, DJ FITZGERALD 1981 2-Chloro-N-[4-methoxy-6-methyl-1,3,5-triazine-2-yl] aminocarbonyl benezenesulfonamide, a new herbicide. J Agric Food Chem 29: 416–424.
4. LINSMAIER EM, F SKOOG 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100–127
5. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
6. Oust Weed Killer 1982 E. I. DuPont deNemours & Co. Inc. Wilmington, DE, Technical Bulletin E-52376.
7. RAY TB 1982 The mode of action of chlorsulfuron: a new herbicide for cereals. Pestic Biochem Physiol 17: 10–17
8. RAY TB 1982 The mode of action of chlorsulfuron: the lack of direct inhibition of plant DNA synthesis. Pestic Biochem Physiol 18: 262–266
9. ROST TL 1984 The comparative cell cycle and metabolic effects of chemical treatments on root tip meristems. III. Chlorsulfuron. J Plant Growth Reg. In press
10. VAN'T HOF J 1968 Control of cell progression through the mitotic cycle by carbohydrate provision. J Cell Biol 37: 773–780
11. WESTERFIELD WW 1945 A colorimetric determination of blood acetoin. J Biol Chem 161: 495–502
12. WHITE PR 1943 Handbook of Plant Tissue Culture. Cattell and Co. Inc., Lancaster, PA, pp 90–116