Differential sensitivity of plant-associated bacteria to sulfonylurea and imidazolinone herbicides

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Abstract

The side effects of sulfonylurea and imidazolinone herbicides on plant-associated bacteria were investigated under pure culture conditions. Eighteen isolates, belonging to the genera *Azotobacter, Azospirillum, Bacillus, Enterobacter Pseudomonas* and *Serratia*, were exposed to four active compounds at concentration ranges similar to those in field soil. The sulfonylureas chlorsulfuron and rimsulfuron inhibited the growth of one of two *Azospirillum* and one of four *Pseudomonas* strains, while the imidazolinones imazapyr and imazethapyr were effective on two out of five *Bacillus* isolates. Surfactants in commercial formulation significantly enhanced rimsulfuron toxicity. With the exception of one *Azospirillum* strain, the differential tolerance of rhizobacteria to these herbicides was related to a differential sensitivity of their target, the activity of the first enzyme in branched-chain amino acid biosynthesis, acetohydroxyacid synthase (AHAS).

Greenhouse pot studies were performed to assess the occurrence of inhibitory effects on bacterial growth in field conditions. Maize seedlings were bacterized with the two strains which had shown in vitro sensitivity to sulfonylureas. Following the application to the soil of a commercial formulation of rimsulfuron at rates of 0, 0.2 and 0.5 μmol a.i. kg^{-1}, significative differences in the resulting degree of bacterial root colonization were found. Moreover, upon co-inoculation with two strains, one tolerant and one sensitive to the herbicide, the presence of rimsulfuron significantly enhanced root occupancy by resistant bacteria, suggesting that shifts in the microbial community structure of crop rhizosphere could indeed result as a consequence of weed control by AHAS inhibitors.

Abbreviations: AHAS–acetohydroxyacid synthase, CETAB– cetyltrimethylammonium bromide, ID$_{50}$–concentration causing 50% inhibition of enzyme activity, LD$_{50}$–concentration causing 50% decrease of growth constant value.

Introduction

Inhibitors of acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also referred to as acetolactate synthase), the first common enzyme of isoleucine, leucine and valine biosynthesis, represent the most active group of herbicidal compounds to date (Stidham, 1991). Two chemically unrelated classes of molecules, the sulfonylureas and the imidazolinones, were developed independently and then both shown to exert phytotoxic activity by interfering with the branched-chain amino acids path (Moberg and Cross, 1990). Several crop-selective derivatives of these compounds have been successfully commercialized as herbicides, selectivity being attributed to rapid metabolic inactivation of the agrichemical in the tolerant crop (Brown, 1990; Tecle et al., 1993).

Experiments carried out with bacterial strains were instrumental in the elucidation of the mode of action of these compounds, providing evidence that their cytostatic activity was mediated by AHAS inhibition (La

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Rossa and Schloss, 1984; Yadav et al., 1986). However, sulfonylureas do not inhibit the growth of wild-type *Escherichia coli* and *Salmonella typhimurium* because of the presence in enteric bacteria of multiple AHAS forms which show differential sensitivity to the herbicides (La Rossa and Smulski, 1984; Yadav et al., 1986). AHAS from all plant species so far examined are, on the contrary, highly sensitive to sulfonylureas (Ray, 1986) and imidazolinones (Singh et al., 1990), whereas very little is still known about the properties of the enzyme from other bacterial sources.

The application of herbicides in agricultural systems may exert side effects on the soil microflora, including a possible shift in microbial community structure (Somerville and Greaves, 1987; Trappe et al., 1984). This may be particularly true in the case of compounds which interfere with amino acid biosynthesis and thus potentially effective also on microbial metabolism. This possibility was investigated for the environmentally safe broad-spectrum herbicide glyphosate (*N*-[phosphono-methyl]glycine) (Grossbard, 1985). At the recommended rate for field usage (0.54 kg a.i. ha^{-1}), this compound initially reduced both bacterial and fungal populations followed by a marked stimulation, which was interpreted as not being caused by the return of the original population, but to the increased activity of a few resistant species (Chakravarty and Chatarpaul, 1990). Conversely, the addition of glyphosate to soil at concentrations ranging from 2 to 200 μg g^{-1} had no significant effect on total bacterial numbers, except at the first week after the treatment where a stimulatory effect was noted (Wardle and Parkinson, 1990). Because of its rapid decomposition by soil microflora (Torstensson, 1985), the possible effect of glyphosate on microbial community structure is thought to be negligible (Grossbard, 1985). Furthermore, a differential sensitivity of the glyphosate target, the activity of the prechorismate pathway enzyme 5-*enol*-pyruvyl-shikimate-3-phosphate synthase, was demonstrated in free-living bacteria (Shulz et al., 1985). To date, for both sulfonylureas and imidazolinones, neither the occurrence of side effects on the bacterial population of the crop rhizosphere nor the existence of a variable bacterial AHAS sensitivity have been adequately investigated.

During the last few decades numerous microorganisms have been shown to exert beneficial effects on plant development. Apart from the well-known case of rhizobial symbionts, free-living rhizosphere bacteria may benefit crops by stimulating plant growth or by reducing the damage from soilborne plant pathogens (Kloepper et al., 1989). Should a herbicidal treatment exert a significant inhibitory effect on root colonization by these plant growth promoting rhizobacteria, there could be a detrimental effect on crop productivity, thus reducing the advantages derived from weed control.

Here we describe the effects of sulfonylurea and imidazolinone herbicides on the growth and AHAS activity of 18 bacterial strains isolated from the rhizosphere of various crops. The occurrence of a possible interference in bacterial root colonization potential was also investigated.

Materials and methods

Chemicals

Unless specified, chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Analytical grade chlorsulfuron (2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl] benzene sulfonamide) and rimsulfuron (*N*-[[(4,6-dimethoxy-2-[(pyrimidinyl) amino]carbonyl]-3-(ethyl sulfonyl)-2-pyridine sulfonamide) were a gift from E.I. DuPont de Nemours; imazapyr ((±)-2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl] – 3-pyridine carboxylic acid) and imazethapyr ((±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl] 5-ethyl-3-pyridinecarboxylic acid) were kindly provided by American Cyanamid.

Strains, media and growth condition

Bacterial strains were isolated from different localities of Tuscany near Florence and Siena from the rhizosphere of various crops (Table 1) and classified by using API tests (Analytab Products Inc., 1979) for Enterobacteriaceae, Bacillaceae and Pseudomonadaceae, and according to the criteria reported in the family of the Azotobacteriaceae for *Azotobacter* and *Azospirillum* (Krieg and Holt, 1984). Bacteria were maintained at 30 ± 1 °C on malate-yeast extract medium (Rodriguez-Caceres, 1982), except for strains belonging to genera *Azotobacter* and *Bacillus*, which were grown on mannitol (Augier, 1956) or glucose-mannitol-lactate (Rennie, 1981) medium, respectively.

Table 1. Strains of rhizobacteria used in the present work

Strain	Species	Source plant
A1	*Azospirillum lipoferum*	Barley
A2	*Azospirillum lipoferum*	Barley
B1	*Bacillus licheniformis*	Wheat
B2	*Bacillus subtilis*	Wheat
B3	*Bacillus megatherium*	Wheat
B4	*Bacillus circulans*	Wheat
B5	*Bacillus pumilus*	Tobacco
E1	*Enterobacter sakazakii*	Wheat
E2	*Enterobacter agglomerans*	Wheat
E3	*Enterobacter cloacae*	Wheat
E4	*Enterobacter cloacae*	Sunflower
N1	*Azotobacter chroococcum*	Wheat
N2	*Azotobacter chroococcum*	Wheat
P1	*Pseudomonas maltophilia*	Wheat
P2	*Pseudomonas vesicularis*	Wheat
P3	*Pseudomonas paucimobilis*	Barley
P4	*Pseudomonas luteola*	Wheat
S1	*Serratia plymuthica*	Barley

Bacterial growth inhibition studies

To evaluate the effect of AHAS-inhibiting herbicides on bacterial growth, four active compounds (the sulfonylureas chlorsulfuron and rimsulfuron, and the imidazolinones imazapyr and imazethapyr) were at first added to the culture medium at concentrations approximating the highest field application rates (3 and 100 μM, respectively).

Bacteria were grown in 12 mL minimal medium (Davies and Mingioli, 1950) containing 1% (w/v) glucose and 0.5 g L^{-1} asparagine in 50-mL erlenmeyer flasks on a rotary shaker (200 rev min^{-1}) at 30 $\pm$ 1 °C. In the case of strains E4, N2, P1 and P2, which did not grow under these conditions, the medium was supplemented with 100 mg L^{-1} yeast extract. Growth was recorded as turbidity at 600 nm. Two independent experiments were performed; means over the two repetitions are presented.

Strains whose generation time was significantly prolonged under these conditions were then grown in the presence of increasing concentrations of the herbicides. At least 8 doses were tested for each compound, ranging from 0.2 to 50 μM for sulfonylureas and from 0.02 to 5 mM for imidazolinones. The concentrations causing 50% growth inhibition (LD$_{50}$) were estimated utilizing the linear regression equations of growth rate

constant values (expressed as percentage of untreated controls) plotted against the logarithm of herbicide concentration. As surfactants in commercial formulation could enhance their action, the effect of the four chemicals on bacterial growth was evaluated again by using commercial formulations (Glean® and Titus®, DuPont; Arsenal® and Pursuit®, Cyanamid) instead of analytical grade compounds. The same doses (referred to the concentration of the active ingredient) were tested, except for sulfonylureas, which ranged from 2 nM to 2 μM.

Acetohydroxyacid synthase assay

AHAS activity was measured in permeabilized bacteria by the method of Jackson (1988) with minor modifications. The reaction mixture consisted of 20 mM potassium phosphate buffer pH 7.5 containing 1 mM MgCl$_2$, 0.1 mM thiamine pyrophosphate, 0.01 mM flavin adenine dinucleotide, 40 mM sodium pyruvate and the cationic detergent cetyltrimethylammonium bromide (CETAB) in a final volume of 0.4 mL. Bacterial strains were grown in minimal medium to the late exponential phase of growth, pelleted and resuspended in 50 mM icecold potassium phosphate buffer pH 7.5 containing 5% glycerol, adjusting cell density to an absorbance of 2.0 at 600 nm. Reaction was started by mixing a suitable aliquot of bacterial suspension to the reaction mixture pre-equilibrated at 35°C. After 5 to 20 min at 35°C, a time during which the reaction was linear, the acetolactate formed was acid-decarboxylated to acetoin, and the latter quantified by the method of Westerfeld (1945). Proper checks were made to quantify not acetolactate-deriving acetoin. The concentration of CETAB had to be modified because preliminary experiments in which AHAS activity was measured in parallel assays containing the detergent at concentrations ranging from 12 μM to 1.6 mM indicated different requirements for an optimal permeabilization of bacterial cells (data not shown). The optimum concentration was then used for each strain (see Results section, Table 3). AHAS specific activity was calculated as described (Jackson, 1988); means over at least four independent determinations are presented.

Enzyme inhibition was estimated by adding to the reaction mixture 0.04 mL of the appropriate solutions of the four active ingredients. At least 8 doses were tested for each compound; for sulfonylureas, concentrations ranged from 2 nM to 20 μM, for imidazolinones from 20 μM to 5 mM; five measurements were performed for each dose. The concentrations causing

Table 2. Growth of rhizobacteria in the presence of sulfonylurea and imidazolinone herbicides

Strain	Generation time (h) [a]				
	Control	Chlorsulfuron (3 μM)	Rimsulfuron (3 μM)	Imazapyr (100 μM)	Imazethapyr (100 μM)
A1	2.9	<u>3.9</u>	<u>6.1</u>	2.8	2.9
A2	2.3	2.4	2.6	2.4	2.4
B1	1.1	1.2	1.1	1.2	1.2
B2	1.2	1.3	1.1	<u>3.4</u>	<u>1.8</u>
B3	0.9	1.0	0.9	0.9	1.0
B4	1.8	1.9	1.9	<u>15.8</u>	2.0
B5	0.9	1.0	1.0	1.1	1.0
E1	0.7	0.7	0.7	0.8	0.7
E2	0.8	1.0	0.9	0.8	0.9
E3	0.7	0.7	0.7	0.7	0.6
E4	3.3	3.6	3.2	3.4	3.6
N1	0.7	0.9	0.7	0.8	0.9
N2	5.2	5.3	5.1	5.1	5.1
P1	0.6	0.6	0.6	0.6	0.6
P2	0.6	0.8	0.7	0.7	0.7
P3	1.9	2.0	1.9	1.8	1.8
P4	2.1	<u>12.6</u>	<u>24.3</u>	2.2	2.0
S1	0.8	0.9	0.9	0.8	0.8

[a] Results are means of two independent experiments. Underlined values differ significantly ($p = 0.05$) from control.

50% inhibition (ID_{50}) of enzyme activity were estimated utilizing the linear regression equations of AHAS activity values (expressed as percentage of untreated controls) plotted against the logarithm of herbicide concentration.

Root colonization experiments

Maize (Zea mays L., F_1 hybrid cv Sandek, Dekalb) seeds were surface sterilized for 30 min in a 50% solution of commercial bleach (3% active chlorine) containing the surfactant Teepol 610 (0.04% v/v, Shell Chemical Co.), thoroughly rinsed with sterile water and allowed to germinate on agarized half strength Hoagland solution (Hoagland and Arnon, 1950). Three days after germination seedlings were inoculated by immersion of the radicle and seminal roots for 30 min in suspensions of bacterial cells harvested during the late exponential phase of growth, resuspended in minimal medium without glucose and turbidometrically adjusted to densities of approximately 10^8 CFU mL^{-1}. Preliminary results demonstrated that incubation for a longer period of time did not increase the number of

adhering cells. In competition experiments, roots were dipped in a mixture of two bacterial strains, each at a density of about 5×10^7 cells mL^{-1}. Introduction of bacteria in this manner ensured uniform initial bacterial populations for all treatments. Roots were then rinsed in sterile water, and seedlings transplanted singly to plastic pots (12-cm diam, 0.65-L vol) filled with a potting mixture (0.8 :1 by weight) of sand:loam (10% organic matter, 0.26% total nitrogen, pH 6.7). A commercial formulation of rimsulfuron (Titus®, DuPont, 25% a.i.) was dissolved in sterile water, and proper dilutions applied to soil samples at a rate of 0.2 or 0.5 μmol kg^{-1} (86 and 215 ppt a.i., respectively); treated samples were hand-mixed for 10 min, and then stored for 8 h before planting, to permit equilibration of herbicide and soil. Plantlets were grown in a growth chamber with a 16-h photoperiod of 500 μE m^{-2} s^{-1} at 25 $\pm$ 1°C; relative humidity was not controlled. They were periodically watered as required with sterile half strength Hoagland solution. On the day of planting, and 3, 7, 10 and 15 days after planting seedlings were harvested, and soil gently removed from roots. The root system was then washed in sterile

Table 3. Activity of acetohydroxyacid synthase from bacteria in the presence of sulfonylurea and imidazolinone herbicides

| Strain | CETAB (mM) | control Uᵃ | Enzyme activity (% of control)ᵇ | | | |
			Chlorsulfuron (30 μM)	Rimsulfuron (30 μM)	Imazapyr (1 mM)	Imazethapyr (1 mM)
A1	0.1	0.39 ± 0.17	0	0	66	45
A2	1.0	0.34 ± 0.10	4	0	8	7
B1	0.1	0.89 ± 0.18	94	96	102	104
B2	0.1	0.13 ± 0.04	95	79	7	15
B3	0.1	0.40 ± 0.03	94	99	99	102
B4	0.1	0.10 ± 0.08	96	76	7	13
B5	0.1	0.69 ± 0.00	98	102	101	103
E1	0.05	2.30 ± 0.58	94	93	104	102
E2	0.05	1.32 ± 0.37	97	96	92	100
E3	0.05	2.15 ± 0.80	97	93	100	94
E4	0.2	0.02 ± 0.01	94	88	98	105
N1	0.2	0.57 ± 0.10	97	88	115	116
N2	0.05	0.75 ± 0.08	102	109	103	107
P1	0.4	0.01 ± 0.00	96	94	98	103
P2	0.05	2.15 ± 0.73	97	91	100	99
P3	0.1	0.11 ± 0.04	96	59	100	101
P4	0.1	0.37 ± 0.07	0	0	83	43
S1	0.05	0.77 ± 0.20	98	101	98	97

ᵃEnzyme activity values, measured by adding in each case to the assay mixture a suitable concentration of the cationic detergent, CETAB, as indicated, are means ± SE of at least four independent determinations.

ᵇData on the effect of the four active compounds on AHAS activity are averaged from at least five replications; underlined values differ significantly ($p = 0.05$) from control.

water to eliminate loosely adhering soil, wiped on sterile blotting-paper, excised from shoots, weighed and macerated with a sterile mortar and pestle in $10 \, mL \, g^{-1}$ of phosphate buffered saline (10 mM potassium phosphate pH 7.5, 100 mM NaCl, 1 mM EDTA) to obtain the first dilution; subsequent 10-fold serial dilutions were prepared, and aliquots (100 μL) plated in duplicate onto malate-yeast extract semiselective medium (Rodrigues-Caceres, 1982). Plates were incubated at 30 ± 1°C for 48 h prior to enumeration of bacterial colonies.

Experiments were first performed to evaluate the effect of rimsulfuron upon root colonization ability of those bacterial strains (A1 and P4) which had previously shown in vitro sensitivity to the herbicide. The experimental design was a randomized complete block with two replicates. Each block comprised 60 pots of the following treatment combinations: two bacterial strains, three rimsulfuron rates (0.2 and 0.5 μmol kg⁻¹, and untreated control), and destructive harvests at 0, 3, 7, 10 and 15 days after seedling inoculation. At

each sampling two plants from each treatment combination were collected from each replicate, resulting in four samples per treatment. The experiment was repeated twice. Since error variances were found to be homogeneous, means over the two repetitions are presented.

Then the effect of rimsulfuron on root colonization was evaluated following co-inoculation with two bacterial strains, one sensitive and one resistant in vitro to the herbicide. Twenty-four entries (2 strain pairs, A1-A2 and P2-P4, combined each with 0, 0.2 and 0.5 μmol kg⁻¹ rimsulfuron, and destructive harvests at 0, 3, 7, and 10 days after bacterization) were evaluated in a completely randomized block design with three replications. At each sampling period, two plants from each treatment combination were collected from each replicate, resulting in six samples per treatment.
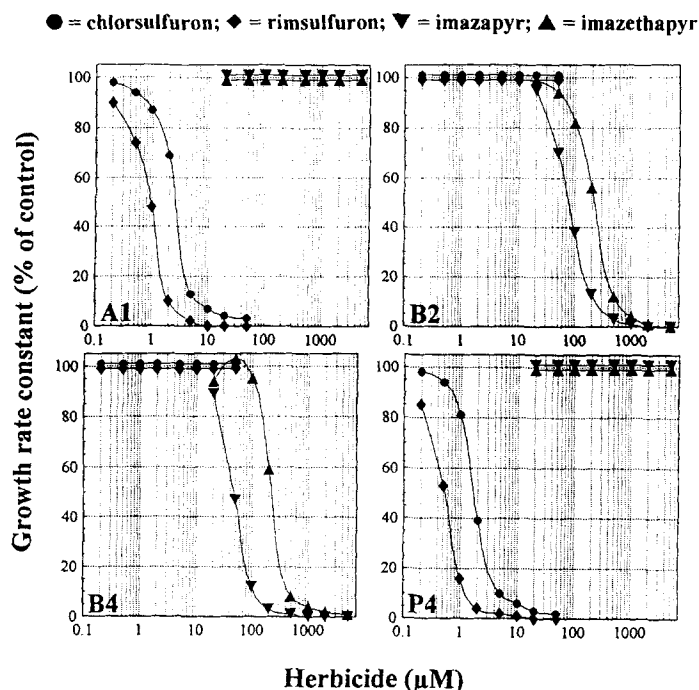
● = chlorsulfuron; ◆ = rimsulfuron; ▼ = imazapyr; ▲ = imazethapyr

Fig. 1. Effect of increasing concentrations of sulfonylurea and imidazolinones herbicides on bacterial growth, expressed as percentage of growth constant value of controls.

Statistical analysis

The data were analysed by using statistical procedures for analysis of variance and t test. Means were separated by the least-significant-difference test. Where differences are reported, they are at the 95% confidence level ($p = 0.05$). Bacterial numbers were subjected to a $\log_{10}$ transformation prior to statistical analysis.

Results

The effects of AHAS-inhibiting herbicides (the sulfonylureas chlorsulfuron and rimsulfuron, and the imidazolinones imazapyr and imazethapyr) on the growth of several rhizobacterial strains are outlined in Table 2. At concentrations approximating the highest field application rates, they resulted in most cases uneffective, being the generation times of only two strains (A1 and P4) out of 18 significantly prolonged by both chlorsulfuron and rimsulfuron, and that of only two other strains (B2 and B4) by imazapyr or imazethapyr.

The results obtained by growing these four sensitive strains in the presence of increasing concentrations of the herbicides indicated a generalized sensi-

tivity towards one of the two classes of agrichemicals. In no case was growth affected by both classes of compounds (Fig. 1). Concentrations causing a 50% decrease of growth constant values (LD_{50}) ranged from 0.5 to 2.6 μM for sulfonylureas and from 47 to 220 μM for imidazolinones. Rimsulfuron and imazapyr were more effective than chlorsulfuron and imazethapyr.

In an attempt to elucidate the mechanism(s) responsible for this differential tolerance, the susceptibility of rhizobacterial AHAS by the herbicides was investigated. Results (Table 3) were on the whole consistent with those previously obtained for bacterial growth, with enzyme activity from 12 of 18 strains tolerant to the herbicides. The only exceptions were the strain P3, in which AHAS activity was inhibited by rimsulfuron and strain A2, in which the enzyme was extremely sensitive to all four compounds, but its growth was completely unaffected (Fig. 2).

Comparison between concentrations causing 50% inhibition of enzyme activity (ID_{50}) and LD_{50} values (Fig. 3) showed good agreement between the effects on bacterial growth and on AHAS activity in the case of imidazolinones. On the contrary, sulfonylureas seemed less effective on cell growth. However, when the effects on bacterial growth were evaluated again by

● = chlorsulfuron; ◆ = rimsulfuron; ▼ = imazapyr; ▲ = imazethapyr
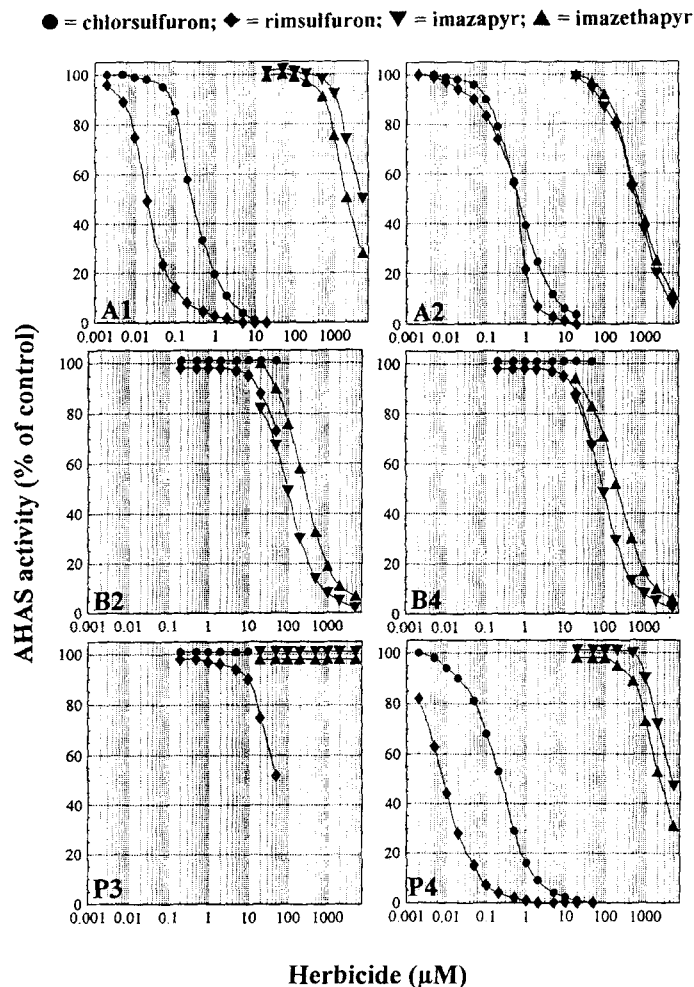

Fig. 2.   Effect of increasing concentrations of sulfonylurea and imidazolinones herbicides on AHAS activity, measured on permeabilized bacteria and expressed as percent of untreated controls.

using commercial formulations instead of analytical grade compounds, the sensitivity to these two agrichemicals increased, with LD_{50} values being very close to ID_{50} values previously found for enzyme activity (Fig. 3).

Root colonization experiments confirmed the sensitivity of strains Al and P4 to rimsulfuron also in soil conditions. The levels of root colonization of maize seedlings following the application of the herbicide are shown in Figure 4. At rates of 0.2 and 0.5 μmol kg^{-1} rimsulfuron significantly slowed down the growth of introduced bacteria in root systems. In both cases there was a decrease in colonization of about half an order of magnitude between 7 and 10 days after the inoculum; then Al achieved a growth similar to that of controls,

while P4 showed a significant inhibition also 15 days after the treatment.

A possible occurrence of side effects of rimsulfuron in agricultural system was suggested also by competition experiments. When maize seedlings were co-inoculated with two bacterial strains, one sensitive and one resistant to the herbicide, the presence of rimsulfuron in the soil significantly promoted root occupancy by the resistant strain (Fig. 5). The effect was particularly appreciable in the case of the pair P2 (tolerant) - P4 (sensitive strain). In control plants the growth of P2 was completely inhibited by P4 root colonization; on the contrary, in treated seedlings P4 inhibition by rimsulfuron allowed the tolerant strain to achieve a higher degree of colonization (Fig. 5).
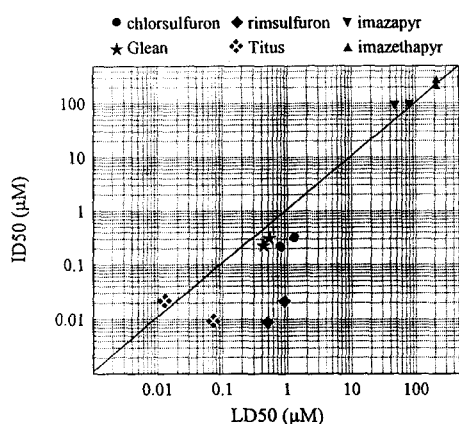
Fig. 3. Comparison between the inhibition brought about by sulfonylurea and imidazolinone herbicides on bacterial growth and on acetohydroxyacid synthase activity. Concentrations causing 50% inhibition of enzyme activity (ID_{50}) and 50% decrease of growth constant value (ID_{50}) were calculated as described in Materials and methods. The effect on bacterial growth was evaluated using both analytical grade compounds and commercial formulations of the herbicides. Data obtained using formulations of imidazolinones, which exactly agreed with those found for the corresponding active principles, are not indicated.
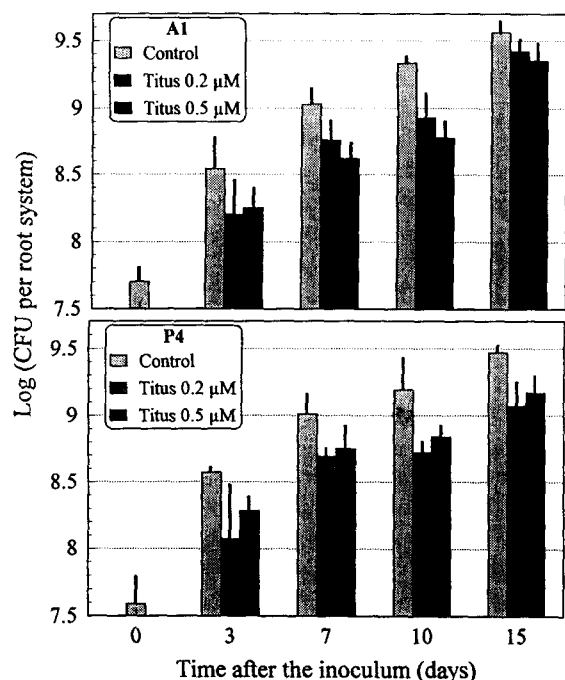

Fig. 4. Effect of rimsulfuron on maize root colonization by strains A1 and P4. Seedlings were bacterized as described in Materials and methods and then transferred into untreated soil, or in soil treated with a commercial formulation of the herbicide at a rate of 0.2 or 0.5 μmol kg^{-1}. At increasing time after the inoculum the resulting bacterial population was evaluated by destructive harvest of the root system and plating onto semiselective medium. Results are mean of two independent experiments.
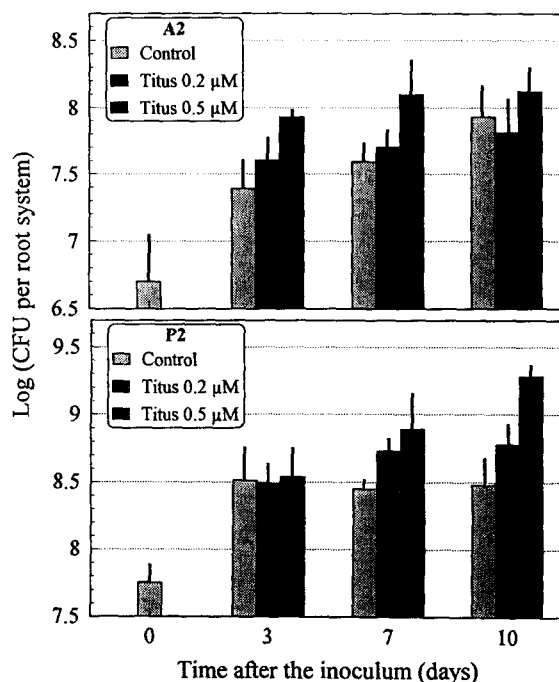

Fig. 5. Effect of rimsulfuron on the competition for maize root colonization between sensitive and tolerant strains. Seedlings were co-inoculated with two bacterial pairs, A1 (sensitive) - A2 (tolerant) and P2 (tolerant) - P4 (sensitive). Plantlets were then transferred into untreated soil, or to soil treated with a commercial formulation of the herbicide at a rate of 0.2 or 0.5 μmol kg^{-1}. At increasing time after the inoculum the resulting bacterial populations were evaluated. Colonies of different strains could be distinguished by differential morphology and dimension. Results presented, mean $\pm$ SD of six replications, refer to root occupancy by tolerant strains.

Discussion

Sulfonylurea and imidazolinone herbicides are extremely potent inhibitors of plant AHAS, which is the reason they are so effective in weed control at exceptionally low rates, typically between 2 to 75 and 10 to 250 g ha^{-1}, respectively (Ray, 1986; Singh et al., 1990). In addition, the target enzyme is present only in plants and microorganisms, so that mammalian toxicity is very low (Beyer et al., 1988; Nakata, 1991). Moreover, these compounds are generally subjected to a relatively fast mineralization by either chemical hydrolysis or microbial degradation (Beyer et al., 1988; Cantwell et al., 1989). In some cases, however, especially under alkaline conditions, residues may persist in soils for several months (Cantwell et al., 1989; Peterson and Arnold, 1985). In the last few years much work has been done to ascertain both the sensitivity of rotational crops and the occurrence of natural variability

of response among inbred lines of sensitive crops to soil residues of these herbicides (e.g. Eberlein et al., 1989; Landi et al., 1989; Peterson and Arnold, 1985). The possible effects of sulfonylureas and imidazolinones on soil microflora had not been investigated to date.

Results from the present study suggest that there is a widespread tolerance of rhizobacteria to these herbicides in that the growth of more than 75% of the examined strains was completely unaffected by high concentrations of all the compounds tested. Moreover, sensitive bacterial strains were 10 to 100-fold more tolerant to both classes of chemicals than cultured plant cells (Forlani et al., 1991; Nakata, 1991; Sathasivan et al., 1991).

In order to investigate the biochemical basis of this differential tolerance, AHAS activity was characterized in the bacterial strains. Tolerance to certain herbicides may be related to a high amount of the target enzyme in the cell. For instance, different levels of AHAS specific activity in roots were shown to be the basis for the differential sensitivity to chlorsulfuron among corn inbreds (Forlani et al., 1991). Rhizobacteria exhibited highly significant differences in AHAS levels (Table 3), but no relationship was detected between this trait and the level of in vivo tolerance. On the contrary, with the exception of strain A2, the sensitivity of AHAS to inhibition by sulfonylureas and imidazolinones was consistent with the differential tolerance of whole cells. No evidence for multiple enzyme forms showing differential sensitivity to the herbicides, as found previously for enterobacteria (La Rossa and Smulski, 1984; Yadav et al., 1986), was obtained: if so, the inhibition curves of AHAS activity in the presence of increasing concentrations of these compounds should display a biphasic profile, but this was not the case (Fig. 2). For strain A2, other mechanisms, such as detoxyfication of the compounds, could be hypothesized to explain the discrepancy between in vivo tolerance and the high sensitivity by the enzyme. However, data obtained in optimizing the procedure for AHAS assay may shed some light on this point. Enzyme activity was measured in permeabilized bacteria as described (Jackson, 1988), but the level of detergent in the assay mixture had to be changed, since preliminary experiments performed with CETAB at various concentrations showed different requirements for an optimal permeabilization (data not shown). The high amount of detergent required to permeabilize A2 cells (Table 3) could indicate that its in vivo tolerance most likely relies upon a relatively low permeability

to the herbicides. This hypothesis is strengthened also by the comparison between the effect of active principles and commercial formulations on bacterial growth. Surfactants in commercial formulations enhanced sulfonylureas effectiveness, especially for rimsulfuron, with LD_{50} values much closer to ID_{50} values found for enzyme activity (Fig. 3), thus suggesting a reduced permeability of these compounds. This behaviour is consistent with previous reports about rimsulfuron activity on weeds (Green and Green, 1993).

Contrary to the widespread sensitivity by AHAS from plants (Ray, 1986; Singh et al., 1990), the enzyme from plant-associated bacteria shows a great variability of response. Moreover, sensitivity to inhibition by the herbicides varies greatly among strains belonging to the same genus, or even to the same species. Even if restricted to a few strains and effective only at relatively high doses, inhibition caused by AHAS inhibitors on rhizobacterial growth is worthy of attention. This sensitivity seems to be more generalized within certain taxonomic groups (for instance, among Azospirillum spp. to sulfonylureas and Bacillus spp. to imidazolinones). Repeated applications of these herbicides could effect a shift in the microbial community structure of the rhizosphere and thus possibly lead to a reduced stimulation of crop yield by beneficial bacteria.

In that results of in vitro experiments may not be consistent with those obtained in the field, where many factors, such as soil particle and colloids, might influence herbicide toxicity, and bacterial strains may exhibit different physiological responses than in pure culture, greenhouse experiments were performed to assess possible effects on bacterial root colonization abilities. The presence of rimsulfuron, the most effective inhibitor of bacterial growth in vitro, caused indeed a significant reduction in root occupancy by both strains A1 and P4 (Fig. 4). The effect was limited, but consistent with data obtained in pure culture experiments, in which the addition of 0.5 μM rimsulfuron to the culture medium did not abolish completely A1 and P4 growth, but reduced it to 6 and 18% of control, respectively (data not shown). In soil conditions, where bacterial growth is much slower, the inhibition could be less severe, and partially relieved by the low concentrations of branched-chain amino acids possibly exuded by root hairs. Moreover, rimsulfuron degrades very rapidly in soil, with a half life of a few days (Bassi et al., 1990). This probably allowed bacterial population to overcome the initial injury, achieving two weeks after the treatment a growth similar to that

of controls (Fig. 4). However, higher rimsulfuron rates could not be tested since they caused a significant inhibition also of maize seedling development (data not presented).

The possibility that shifts in the microbial community structure of crop rhizosphere do actually result as a consequence of weed control by AHAS inhibitors is strengthened by the results of competition trials. Co-inoculation in control maize seedlings of two *Azospirillum* or *Pseudomonas* isolates, one resistant and one sensitive in vitro to rimsulfuron, resulted in both cases in a prevalent root occupancy by sensitive bacteria (data not shown). When soil was treated with the herbicide, growth inhibition by rimsulfuron, even if limited, allowed tolerant strains to compete with the formers, achieving significantly higher densities in the root system (Fig. 5). The effect is particularly evident in the case of the tolerant strain P2, whose growth in the absence of the herbicide was completely inhibited by P4 root colonization.

Although a generalization is not advisable, and confirmative field trials should be performed, it is concluded from these results that at recommended rates of application sulfonylurea and imidazolinone herbicides can influence the microbial structure of the rhizosphere. As the use of these herbicides is expected to increase because of their effectiveness for weed control and low toxicity to mammals, field applications should be preceded by proper tests in order to identify and avoid negative impacts on soil microflora, expecially when seed inocula with plant-growth-stimulating bacteria are carried out to enhance crop yield.

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References

Analytab Products Inc. 1979 Analytical profile index: Enterobacteriaceae and other gram-negative bacteria. API Division of Ayer Labs, Plainview, NY.

Augier J 1956 A propos de la numeration des *Azotobacter* en milieu liquide. Ann. Inst. Pasteur 91, 759–765.

Bassi A, Bencivelli A, Fabiani G P, Gamberini C, Massasso W, Salomone M C and Turchiarelli V 1990 DPX-E9636 - Nuovo graminicida di post-emergenza del mais ad ampio spettro e ridotta persistenza. Atti Giorn. Fitopatol. 1, 125–132.

Beyer E M, Duffy M J, Hay J W and Schlueter D D 1988 Sulfonylurea herbicides. *In* Herbicides: Chemistry, Degradation and Mode of Action. Eds. P C Kearney and D D Kauffman. Vol. 3, pp 117–189. Marcel-Dekker, New York, NY.

Brown H M 1990 Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. Pestic. Sci. 29, 263–281.

Cantwell J R, Liebl R A and Slife F W 1989 Biodegradation characteristics of imazaquin and imazethapyr. Weed Sci. 37, 815–819.

Chakravarty P and Chatarpaul L 1990 Non-target effect of herbicides. I. Effect of glyphosate and hexazinone on soil microbial activity. Microbial population, and in-vitro growth of ectomycorrhizal fungi. Pestic. Sci. 28, 233–241.

Davies B D and Mingioli E S 1950 Mutants of *Escherichia coli* requiring methionine or vitamin B$_{12}$. J. Bacteriol. 60, 17–28.

Eberlein C V, Rosow K M, Geadelmann J L and Openshaw S J 1989 Differential tolerance of corn genotypes to DPX-M6316. Weed Sci. 37, 651–657.

Forlani G, Nielsen E, Landi P and Tuberosa R 1991 Chlorsulfuron tolerance and acetolactate synthase activity in corn (*Zea mays* L.) inbred lines. Weed Sci. 39, 553–557.

Green J M and Green J H 1993 Surfactant structure and concentration strongly affect rimsulfuron activity. Weed Technol. 7, 633–640.

Grossbard E 1985 Effects of glyphosate on the microflora: with reference to the decomposition of treated vegetation and interaction with some plant pathogen. *In* The Herbicide Glyphosate. Eds. E Grossbard and D Atkinson. pp 159–185. Butterworth and Co., London, UK.

Jackson J H 1988 Rapid assay of acetolactate synthase in permeabilized bacteria. Methods Enzymol. 166, 230–233.

Kloepper J W, Lifshitz R and Zablotowicz R M 1989 Free-living bacteria inocula for enhancing crop productivity. Trends Biotechnol. 7, 39–44.

Krieg N R and Holt J G 1984 Bergey's Manual of Systematic Bacteriology, Vol 1. pp 94–104. Williams and Wilking, Baltimore, USA.

La Rossa R A and Schloss J V 1984 The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. 259, 8753–8757.

La Rossa R A and Smulski D R 1984 *IlvB*-encoded acetolactate synthase is resistant to the herbicide sulfometuron methyl. J. Bacteriol. 160, 391–394.

Landi P, Vicari A and Catizone P 1989 Response of maize (*Zea mays* L.) inbred lines and hybrids to chlorsulfuron. Weed Res. 29, 265–271.

Moberg W K and Cross B 1990 Herbicides inhibiting branched-chain amino acid biosynthesis. Pestic. Sci. 29, 241-246.

Nakata M 1991 The mode of action of chlorsulfuron in culture cells of tobacco and hamster. J. Pestic. Sci. 16, 583–590.

Peterson M A and Arnold W E 1985 Response of rotational crops to soil residue of chlorsulfuron. Weed Sci. 34, 131–136.

Ray T B 1986 Sulfonylurea herbicides as inhibitors of amino acid biosynthesis in plants. Trends Biotechnol. Sci. 11, 180–183.

Rennie J 1981 A single medium for the isolation of acetylene reduction (dinitrogen fixing) bacteria from soil. Can. J. Microbiol. 27, 8–14.

Rodriguez-Caceres E A 1982 Improved medium for isolation of *Azospirillum* spp. Appl. Environ. Microbiol. 44, 990–991.

Sathasivan K, Haughn G W and Murai N 1991 Molecular basis of imidazolinone herbicide resistance in *Arabidopsis thaliana* var Columbia. Plant Physiol. 97, 1044–1050.

Schulz A, Krüper A and Amrhein N 1985 Differential sensitivity of bacterial 5-enolpyruvyl shikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiol. Lett. 28, 297–301.

Singh B K, Newhouse K E, Stidham M A and Shaner D L 1990 Imidazolinones and acetohydroxyacid synthase from plants. *In* Biosynthesis of Branched-Chain Amino Acids. Eds. Z Barak, D M Chipman and J V Schloss. pp 357–371. WCH Publishers, Weinheim, Germany.

Somerville L and Greaves M P V 1987 Pesticide effects on soil microflora. Taylor and Francis, London, UK.

Stidham M A 1991 Herbicides that inhibit acetohydroxyacid synthase. Weed Sci. 39, 428–434.

Tecle B, DaCunha A and Shaner D L 1993 Differential routes of metabolism of imidazolinones, basis for soybean (*Glycine max*) selectivity. Pestic. Biochem. Physiol. 46, 120–130.

Torstensson L 1985 Behaviour of glyphosate in soils and its degradation. *In* The Herbicide Glyphosate. Eds. E Grossbard and D Atkinson. pp 137–150, Butterworth and Co., London, UK.

Trappe J M, Molina R and Castellano M 1984 Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Ann. Rev. Phytopathol. 22, 331–359.

Wardle D A and Parkinson D 1990 Influence of the herbicide glyphosate on soil microbial community structure. Plant and Soil 122, 29–37.

Yadav N, McDevitt R E, Benars S and Falco S C 1986 Single amino acid substitution in the enzyme acetolactate synthase confers resistance to the herbicide sulfometuron methyl. Proc. Natl. Acad. Sci. USA 83, 4418–4422.

Westerfeld W W 1945 A colorimetric determination of blood acetoin. J. Biol. Chem. 161, 495–502.

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