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Toxicity of 2,4-Dichlorophenoxyacetic Acid to *Rhizobium sp* in Pure Culture

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2,4-Dichlorophenoxyacetic acid (2,4-D) and other phenoxyalkanoic herbicides have been used as aquatic and terrestrial herbicides since the late 1940s. They are highly effective against selected broadleaf plants and have found applications in a wide variety of situations, from weed control in lawns, gardens, cereal crops and pastures to defoliants in forestry and warfare (Sinton et al. 1986). The fact that large quantities of 2,4-D and related phenoxyalkanoic herbicides are manufactured and applied each year in Argentina and in other countries, indicates the importance of having effective means of treating production wastes as well as a thorough understanding of the fate and impacts of these chemicals in the environment. Thus, it is important to investigate the effects that this herbicide might have on soil microorganisms, since they are the principal agents involved in the natural cycling of surficial organic matter. Whether free-living or occurring in symbiotic association with higher plants, they can be also crucial in maintaining soil fertility by conversion of atmospheric nitrogen to forms available for plants.

Soil bacteria, referred to as rhizobia belonging to the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium,* have the unique ability to induce nitrogen-fixing nodules on the roots or stems of leguminous plants. The symbiotic interaction is host specific, but peanuts (*Arachis hypogaea*) are infected by a variety of rhizobia and these have not yet been given species names. Degree of pesticidal inhibition of rhizobia varies between strains. Although in a large variety of microorganisms (such as *Pseudomonas, Arthrobacter, Xanthobacter* and *Alcaligenes* species) it has been demonstrated adaptability to 2,4-D and other xenobiotics by succesive exposure to these compounds (Sinton et al.,1986; (Ditzelmuller et al., 1989), as far as we know, the adaptation of *Rhizobium* to the presence of 2,4-D has not been reported. Studies in our laboratory revealed that *1* mM *2,4-D* affects the growth of *Rhizobium sp* M4 (able to nodulate *Arachis hypogaea*) in a reversible way, producing also biochemical changes that alterate membrane fluidity (Fabra et al. 1987, 1992). We previously also demonstrated that parent 2,4-D is incorporated in this bacterium by a constitutive and energy-independent transport system (Arias and Fabra, 1993).

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Xenobiotics are foreign chemicals that interact with macromolecules in a variety of ways. These interactions are usually reversible in nature and occur via noncovalent reactions. However, many chemicals induce toxicity by a covalent binding to the cell macromolecules. It was well reported that halogenated aromatic compounds represent a major class of compounds able to bind protein covalently (Hinson and Roberts, 1992), but little is known about the nature of 2,4-D toxicity.

On the basis of our previous results, the present study was designed to determine the following aspects of 2,4-D toxicity on *Rhizobium sp :*
a) The influence of 2,4-D on stationary-phase *Rhizobium sp* culture, the adaptation of this bacteria to an inhibitory herbicide concentration, and the possibility that it was used as a source of energy in minimal media.
b) The herbicide distribution in this prokaryotic cell, as well as the role that the cell wall plays in the 2,4-D incorporation.
c) 2,4-D irreversible binding to macromolecules such as lipids and proteins.

MATERIALS AND METHODS

2,4-Dichlorophenoxy (2-^{14}C)acetic acid was obtained from Amersham Corp. Oakville, Ontario, Canada. All other reagents were purchased from Sigma Chemical Corp.,St. Louis, Missouri, U.S.A.

Rhizobium sp M4, isolated from peanuts (*Arachis hypogaea*) nodules, was used in this study. It was kindly provided by Dr. Lilian Frioni, Universidad Nacional de Rio Cuarto. Stock cultures were maintained on yeast extract mannitol-agar (YMA) slants (Vincent 1970) and transferred regularly. Growth studies were done as follows: Cells were grown at 28 ± 2°C in 500 ml Erlenmeyer flasks containing 250 ml yeast extract-mannitol medium (YEM) (Vincent 1970) in an incubator shaker. Treated media were obtained by the addition of 0.25 ml of a 1M 2,4-D/ethanol solution (0.22 µg 2,4-D and 1 µl ethanol per ml media) or 0.625 ml of the same solution (0.55 µg 2,4-D and 2.5 µl ethanol per ml media) in order to give a final herbicide concentration of 1 mM or 2.5 mM respectively. Control flasks had the equivalent volume of ethanol without 2,4-D. Growth was monitored as optical density at 620 nm in a Varian Techtron spectrophotometer. To study the adaptation of the bacteria to 2.5 mM of 2,4-D (a lethal herbicide concentration to *Rhizobium sp* M4) they were grown with or without 1 mM herbicide for 24 h and transferred to a culture medium containing 2.5 mM 2,4-D. The growth was followed measuring the cellular density as it was described above. To determine the ability of *Rhizobium sp* to utilize 2,4-D as the only energy source, cells were grown in a minimal medium (pH 6.8) containing PO_4HNa_2 2.9 g L^{-1}, PO_4H_2K 1.5 g L^{-1}, NaCl 0.25 g L^{-1}, NH_4Cl 0.5 g L^{-1}, $MgSO_4$ 0.12 g L^{-1}, $CaCl_2$ 1 g L^{-1} and 1 mM 2,4-D. The absorbance was measured as described above.

In order to know 2,4-D distribution in *Rhizobium sp* and the effect of the pregrowth in 1mM 2,4-D supplemented medium on this parameter, cells were grown for 12 h in control or treated (1mM 2,4-D) medium. Bacteria were harvested by centrifugation at 4,000 x g and cellular pellets were washed with

phosphate-buffered saline (PBS)and resuspended in 8 ml of the same solution. Then 0.5 µCi ^{14}C-2,4-D (157 µCi /mmol) was added, and after 30 min of incubation, cells were centrifuged and washed twice with PBS. To obtain the cellular fractions (cytosol, cell membrane and wall) the method was applied as described by Kaback, 1971. Radioactivity in each fraction was determined using a Beckman LS-1000 liquid scintillation system. Scintillant contained 0.2 g of 1,4-bis(2,4-methylphenyloxozolyl)benzene and 4 g of 2,5-diphenyloxozole per litre of triton (1:3, V/V).

Spheroplasts, for the determination of 2,4-D uptake, were obtained from control or treated cultures in late logarithmic phase following the method described by Kaback, H., 1971. They were resuspended in 3 ml of phosphate buffer (pH 6.6) with 20 % sucrose and 20 mM $MgSO_4$ (osmolarity -5.89 atm). Uptake was initiated by the incubation at 28 ± 2°C of the resuspended spheroplasts and by the addition of 0.5 µCi ^{14}C-2,4-D (157 µCi/mmol). Samples (1 ml) were taken after 3, 7 and 15 min of incubation and the radioactive incorporation was stopped by the addition of 5 ml of phosphate buffer at 4°C. These samples were quickly centrifuged and washed twice. Radioactivity was determined as explained above.

Irreversible binding of 2,4-D to *Rhizobium sp* proteins and lipids was determined in 12 h control or treated (1 mM 2,4-D) cultures. They were harvested by centrifugation at 4,000 x g and cellular pellets were washed with PBS, resuspended in 8 ml of the same solution and incubated at 28 ± 2°C during 60 min with 0.5 µCi ^{14}C-2,4-D (157 µCi /mmol). Irreversible binding to proteins was determined by the method of Rao and Recknagel, 1969. Proteins were precipitated by the addition of 10% and then 5% cold trichloroacetic acid (TCA). The precipitate was washed successively with ethanol: ether: chloroform (2:2:1, V/V/V), then with acetone and ,finally, with diethyl ether. The dry protein residue was weighed and then, an aliquot was dissolved in concentrated formic acid and radioactivity counted. Irreversible binding to lipids was determined by the method of Castro and Diaz,l972, adding chloroform:methanol (2:1, V/V) to the cellular pellet. The mixture was centrifuged for 10 min at 3,000 x g and the supernatant was transferred and partitioned with chloroform:methanol:water. The two phases were centrifuged at 3,000 x g (5 min) and the lower phase was evaporated under a nitrogen atmosphere. The residue was weighed and the radioactivity was determined.

Protein was determined by the method of Bradford, 1976, using bovine serum albumin as standard.

All the experimental differences were compared by the Student's *t*-test. Level of statistical significance accepted was $p < 0.05$.

RESULTS AND DISCUSSION

The deleterious effect of 1 mM 2,4-D on *Rhizobium sp* growth when it was present from the beginning of the incubation has been reported in a previous work (Fabra et al. 1987). However, the results in Fig. 1 demonstrate that when 1

mM 2,4-D is added to cultures in stationary phase (at 12 h of incubation), the effect of the herbicide is more drastic, in such a way that a diminution in the optical density is observed, indicating a process of cell lysis. The lysis was confirmed by plating the stationary-phase culture at the end of incubation. The number of colony forming units observed after 5 days of incubation were evidently lower than those observed in plates inoculated with control culture (data not shown). This result could be a consequence of pH differences in the medium, since pH of culture medium dropped from 6.8 to 5 after 12 h of incubation. As 2,4-D is a relatively strong acid (pKa 2.9) the ionized form increased as the pH increased. The opposite occurred in the case of the nonionized form (Erne 1966). The nonionized forms are believed to be more readily transported across the cell membrane (Sinton et al. 1986) and thus, 2,4-D cell concentration might be higher in this condition. Bergesse and Balegno (1995) investigated the effect of pH on 2,4-D uptake in Chinese hamster ovary cells. These authors demonstrated that 2,4-D influx was higher at pH 4.5 than at pH 8.5. However, we cannot overlook the possibility that *Rhizobium sp* 2,4-D sensitivity was increased at the stationary-phase of growth as a result of its different biochemical composition compared with exponential-phase of growth.

Although we have previously demonstrated that it is possible to reverse the adverse effect produced by 1 mM 2,4-D on *Rhizobium sp* growth by changing treated bacteria to a control medium (Arias and Fabra 1993) when 1 mM 2,4-D-grown cells were transferred to a medium containing 2.5 mM 2,4-D, growth was not observed, as judged by the turbidity of the flask measured (Fig. 2). The same result was obtained when control bacteria were placed into a 2.5 mM 2,4-D medium (data not shown). These results demonstrated that 2.5 mM 2,4-D is a lethal concentration to this microorganism and that the pregrowth of *Rhizobium sp* in 1 mM 2:4-D-medium during 12 h is not enough to produce adaptation to lethal 2,4-D concentration. Knowing that bacteria in unmixed culture have limited adaptative capacity (Reanney et al. 1982), the results obtained are not unexpected.

A large variety of bacteria belonging to different genera and capable of degrading 2,4-D and related compounds have been isolated (Sinton et al. 1986, Ditzelmuller 1989, Sandmann and Loos 1987). However, no data about 2,4-D degradative capacity in rhizobia are available. The ability of *Rhizobium sp* cells to utilize 2,4-D as a sole source of carbon was studied by incubating cells in minimal media containing 1 mM 2,4-D. Fig. 3 shows that *Rhizobium sp* cells were capable of growing under these conditions, demonstrating that 2,4-D is used as a carbon and energy source. These results agreed with those reported by Comeau et al. *(1993)* in *Pseudomonas cepacia.* The long lag observed when *Rhizobium sp* is growing in a medium with 2,4-D as the only carbon and energy source could be indicating that an acclimatization period for 2,4-D degradation was necessary. Another possibility is that 2,4-D produces a stronger effect on the bacteria growth when they are in a minimal medium than when they are in a rich one. Leslie Grady (1985) classified the xenobiotic metabolism in “gratuitous” and “co-metabolism”. The last is defined as the transformation, by an unmixed culture, of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound. Microorganisms that co-metabolize substrates convert them to organic products without obtaining a

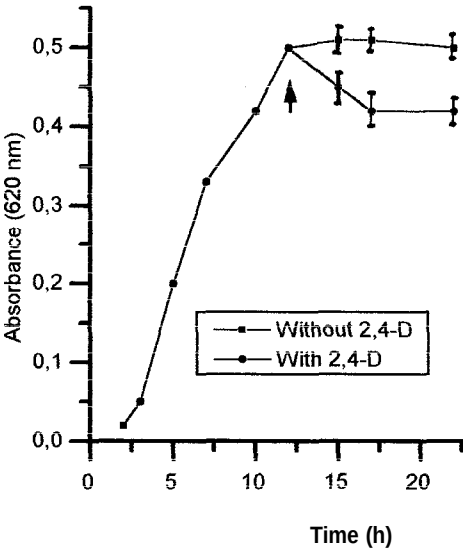

Figure 1. *Effect of the addition of 2,4-D to cultures in stationary growth phase.* **Arrow indicates the addition of 2,4-D. Data are** means **± S.E. of 3 determinations.**

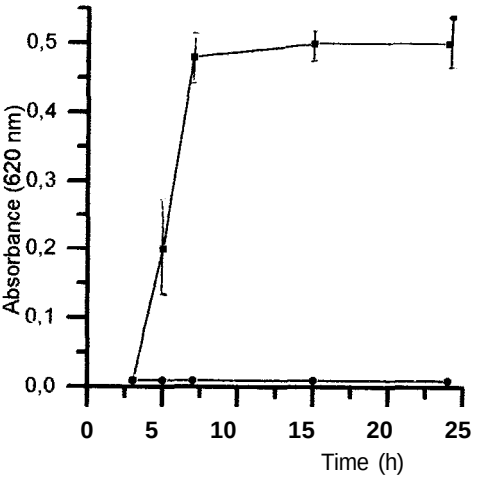

Figure 2. *Effect of 2.5 mM 2,4-D on the bacterial* growth. ■ Inocula **fom a 1 mM 2,4-D medium placed into 1 mM 2,4-D medium. ◆ Inocula from a 1 mM 2,4-D medium placed into 2.5 mM medium. Data are means ± S.E. of 3 determinations.**

significant amount of carbon or energy, and no growth appears to be associated with co-metabolic substrate utilization. The biodegradation of xenobiotics by gratuitous metabolism is mediated by an existing enzyme that metabolizes standard substrates, which happens to have suitable catalytic activity toward a novel substrate. In *Arthrobacter* sp it was demonstrated that low molecular weight aromatic compounds act as inducers for the 2,4-D degrading enzyme system (Sandmann and Loos, 1988). It is possible to assume that 2,4-D is degraded by this gratuitous mechanism in *Rhizobium sp* under these experimental conditions. However, further studies are necessary to confirm this presumption.

We have previously reported that 1 mM 2,4-D-grown cells were then able to incorporate 12 - 15 times more ^{14}C-2,4-D compared with cells that had grown in a 2,4-D-free media (Fabra et al.1987). We also determined that the herbicide is incorporated in *Rhizobium sp* via a constitutive and energy-independent transport system (Arias and Fabra 1993). As the radioactivity from ^{14}C-2,4-D was determined in cellular pellets, its localization in the cell was unknown. It was therefore of interest to determine if the herbicide penetrates to the cell's interior and combines with cell constituents interfering thereby with their normal functions. From the results presented in Table 1, it is clear that 2,4-D went through the cellular envelope of control (pregrowth without 2,4-D) and treated (pregrowth with 1mM 2,4-D) *Rhizobium sp,* being present in the cytosol. The highest concentration was always found in the cell wall, suggesting that it acts as a permeability barrier for 2,4-D intracellular incorporation. Although 2,4-D concentrations in cell membranes were higher in treated cells than in the

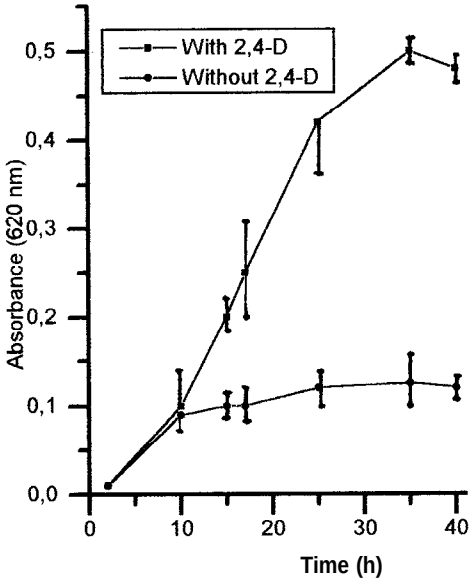

Figure 3. *2,4-D as the only carbon and* energy source. Data are means ± S.E. of 3 determinations.

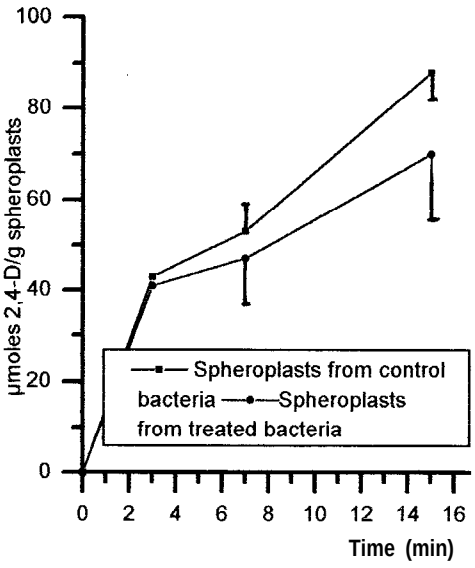

Figure 4. *2,4-D uptake in spheroplasts.* Data are means ± S.E. of 3 determinations.

controls, there was no difference in total radioactivity determined in cellular fractions from control or treated bacteria. This result seems to be inconsistent with the higher ^{14}C-2,4-D uptake previously determined in the cellular pellet from treated bacteria (Fabra et al. 1987). However, this discrepancy might be explained by a 2,4-D accumulation in the periplasmic space of 2,4-D pregrowth cells, since radioactivity was also found in the washings. In order to obtain more evidence that the herbicide is accumulated in *Rhizobium sp* periplasmic space and that its wall acts as a permeability barrier for 2,4-D incorporation, the herbicide uptake in spheroplasts was measured.

Table 1. 2,4-D distribution in *Rhizobium sp* cells.

Treatment	(µmoles 2,4-D/g cellular pellet)		
	Wall	Membrane	Cytosol
Control	7.97 ± 0.67	1.60 ± 0.26	1.00 ± 0.30
1 mM 2,4-D	7.80 ± 1.15	2.70 ± 0.63*	1.70 ± 0.50

Data are means ± S.E. of 3 determinations *p<0.05

As shown in Fig. 4, spheroplasts 2,4-D content (almost 70 µmoles/g) is higher than that obtained addend the µmoles of 2,4-D found in all the cellular fractions (almost 10 µmoles/g, data shown in Table 1). Since in spheroplasts cell wall is not present, this result might be confirming the hypothesis that *Rhizobium sp* cell wall is able to retain, at least in part, the herbicide. However, in view of our previous results (Fabra et al. 1987, 1992, Arias and Fabra. 1993) the intracellular concentration that 2,4-D reaches in *Rhizobium sp* cell is enough to

produce the harmful effects observed. It is also possible to observe in Fig. 4 that no differences between the uptake in spheroplasts obtained from control or treated bacteria were determined. This result is supporting the presumption that, in 2,4-D pregrowth cells, ^{14}C 2,4-D accumulates in periplasmic space.

In an attempt to investigate if 2,4-D combines with cell macromolecules, irreversible binding to proteins and lipids were determined. Irreversible binding of xenobiotic to macromolecules is considered of potential relevance in their toxicological effects (Villarruel et al 1975). Covalent binding of a drug or metabolite to macromolecules is defined by the following characteristic: binding is irreversible; that means that macromolecules retain the radiolabel after repeated and stringent washings with both polar and nonpolar solvent. In this study, ^{14}C-2,4-D binding to lipids and proteins has been demonstrated to be irreversible, according to the above criteria, without elucidation of the structural nature of drug-molecule bond. We determined the formation of 2,4-D-protein and 2,4-D-lipid covalent adducts and no difference between control or pre-treated bacteria was found (Table 2). The fact that 2,4-D-protein adduct was mainly formated in both groups would be demonstrating a direct toxic 2,4-D effect on *Rhizobium sp* if the target protein is a specific one with a physiological function or a catalytical activity that may be impaired. Thus, for mechanistic studies, the elucidation of the specific 2,4-D target may be desirable and more studies should be done in this sense.

Table 2. 2,4-D irreversible binding to *Rhizobium sp* proteins and lipids.

Treatment	(nmoles 2,4-D/g cellular pellet)	
	Lipids	Proteins
Control	0.16 ± 0.08	4.97 ± 0.88
1 mM 2,4-D	0.16 ± 0.06	5.60 ± 1.10

Data are means ± S.E. of 3 determinations.

Taken together, the results of this investigation have begun to clarify the mechanism by which the herbicide produces its toxic effects in *Rhizobium sp.* Although bacterium cell wall might act as a permeability barrier for 2,4-D incorporation, the herbicide goes through the cell envelope and is present in the cytosol. The concentration reached in the cell is enough to produce the toxic effect reported in this and in previous works. It is possible that toxicity would be mediated by its ability to combine with cellular macromolecules, interfering in this way with cell's normal functions.

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