Rev. Int. Contam. Ambie. 31 (3) 227-236, 2015

GENOTOXIC EFFECT OF AZINPHOS METHYL IN BACTERIA AND IN HUMAN LYMPHOCYTE CULTURES AFTER PLANT ACTIVATION

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(Recibido agosto 2014; aceptado enero 2015)

Key words: azinphos methyl, *Salmonella typhimurium*, human lymphocyte cultures, plant metabolism, sister chromatid exchange

ABSTRACT

The evaluation of the potential risk of pesticides applied to crops consumed by humans in Mexico is appropriate and necessary because plant pro-mutagenic transformation in toxic metabolites and their subsequent incorporation involve a risk for health when such crops are ingested. Plant metabolism of agricultural insecticides produces compounds that could be introduced in the food chain, increasing the contamination and poisoning risk by agrochemical metabolism. In this study we evaluated the effect of the organophosphorus insecticide azinphos methyl transformed by S10 fraction of broad bean (*Vicia faba*), using as indicator of mutagenic damage the reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 and the sister chromatid exchange (SCE) in human lymphocyte cultures. Results of mutagenicity showed that when *Salmonella* TA98 and TA100 strains were treated directly with azinphos methyl, negative response was obtained. The same occurred with human lymphocytes tested directly with this insecticide. When *Vicia faba* S10 enzymatic mix was added, there was a mutagenic response in both *Salmonella* strains. These results suggest that the mechanisms to induce mutations by azinphos methyl were frameshift mutation (TA98 strain) as well as pair bases substitution (TA100 strain). Likewise, SCE production was significant and dose-response relationship was observed in human lymphocyte cultures. The cell kinetics (M1, M2 and M3 cells), the replication index and the mitotic index are also analyzed. Only in the treatments with S10 fraction the effects were observed. At the highest concentration mitotic inhibition was produced.

Palabras clave: azinfos metílico, *Salmonella typhimurium*, cultivo de linfocitos humanos, metabolismo de plantas, intercambio de cromátidas hermanas

RESUMEN

Es conveniente y necesaria la evaluación del riesgo potencial de la aplicación de plaguicidas a los cultivos de consumo humano en México debido a que las plantas a través de la activación metabólica son capaces de transformar promutágenos en metabolitos

tóxicos y su subsecuente incorporación involucra un riesgo para la salud cuando tales cosechas se ingieren. El metabolismo de insecticidas agrícolas produce compuestos que pueden introducirse en la cadena alimenticia, con lo que se incrementa la contaminación y el riesgo de envenenamiento por la transformación de los agroquímicos. En este estudio se evaluó el efecto del insecticida organofosforado azinfos metílico transformado por la fracción S10 del haba (*Vicia faba*). Se utilizó como indicador de daño la mutación reversa en las cepas TA98 y TA100 de *Salmonella typhimurium* y el intercambio de cromátidas hermanas (ICH) en cultivo de linfocitos humanos. Los resultados de mutagenicidad mostraron que cuando las cepas TA98 y TA100 de *Salmonella* fueron tratadas directamente con azinfos metílico se obtuvo una respuesta negativa, lo mismo ocurrió con los linfocitos humanos al aplicarles directamente este insecticida. Cuando se agregó la mezcla enzimática S10 de *Vicia faba* junto con el azinfos metílico, hubo respuesta positiva en ambas cepas. Este resultado sugiere que el mecanismo que induce mutaciones, lo hace por corrimiento del marco de lectura (cepa TA98), así como por sustitución de pares de bases (cepa TA100). Asimismo, la inducción de ICH fue significativa y se obtuvo una relación de concentración-respuesta en los linfocitos humanos en cultivo. Adicionalmente, se analizó la cinética de proliferación celular (células M1, M2 y M3), el índice de replicación y el índice mitótico. Únicamente en los tratamientos con S10 se observaron efectos y en la concentración más alta hubo inhibición de la mitosis.

INTRODUCTION

The fact that plants undergo enzymatic transformation makes a deeper analysis necessary, since persons are exposed to pesticides directly not only when they are applied to vegetables, but also through the metabolites that are stored in different structures which may turn out to be more dangerous. Plants can bio-concentrate these environmental agents and convert pro-mutagens into toxic metabolites (Plewa *et al.* 1988, 1993, Wagner *et al.* 2003). This fact raises the concern that plant systems might also activate agrochemicals and environmental agents, thereby introducing new mutagens into the human food chain (Plewa 1978).

Peroxidases are among the most important enzymes for oxidative metabolism in plants (Sandermann *et al.* 1982, Wildeman and Nazar 1982). Peroxidases are relatively stable enzymes and are abundant in homogenates of plants. They are capable to catalyze two categories of oxidative reactions in plant cells, the classical peroxidative reaction that need H_2O_2, and an oxidative reaction that requires molecular oxygen (Lamoureux and Frear 1979) originated free radical intermediates that interacted with DNA and increased the mutagenicity of some chemicals (Lamoureux and Frear 1979, Donh and Krieger 1981, Plewa *et al.* 1993). The hydrolysis is catalized by some hydrolases, esterases, amidases, O-alkylhydrolases, etc. and represents a general degradation mechanism of xenobiotics in animals, microorganisms and plants (Menn 1978, Shimabukuro *et al.* 1982).

In animals and plants the last step of molecular biotransformation is conjugation (Higashi 1988). The conjugates are excreted by animals, but in plants there is not an excretory system, so plants polymerize and incorporate these conjugates into their structural components in a way that the initial products, the reactive intermediates (and/or active oxygen species) and the final products may cause, first, damage to the plant itself, and second, if conjugates are stored in the same plant, they may be set free in the gastrointestinal tract or in the organs of animals when plants are eaten. The last event could specifically occur as a consequence of pesticide application to edible plants (Sandermann 1988).

Vicia faba is highly sensitive to pesticide effects (Gómez-Arroyo and Villalobos-Pietrini 1995, Gómez-Arroyo *et al*. 1995, Valencia-Quintana *et al*. 1998) and is considered metabolically active because it contains in the roots and in other tissues the S10 enzymatic fraction, capable of metabolizing or activating different compounds (*in vitro* activation) as well as the extracts prepared from promutagen-treated roots (*in vivo* activation; Takehisa and Kanaya 1983, Takehisa *et al*. 1988, Calderón-Segura *et al*. 1999, Gómez-Arroyo *et al*. 2000a, Flores-Maya *et al*. 2005).

The *Salmonella typhimurium* assay (*Salmonella* test, Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage leading to gene mutations (Mortelmans and Zeiger 2000).

The Ames test utilizes specific strains of the bacteria *Salmonella typhimurium* as tools to detect mutations.

These strains are known as auxotrophs because they cannot synthesize the amino acid histidine and thus they will not grow unless the nutrient is supplied in growth media. This test determines the ability of a particular substance to cause a reverse mutation of these auxotrophs to the original prototrophic state. The mutant colonies that can synthesize histidine are called revertants and the test is often referred to as a "reversion assay" (Mortelmans and Zeiger 2000). Several strains of the *S. typhimurium* may be used for testing. Among the most frequently used are TA98, which identifies frameshift mutagens, and TA100, which detects mutagens that can cause the substitution of pair bases. Each of these mutations is designed to be responsive to mutagens that act by different mechanisms (Maron and Ames 1983).

The sister chromatid exchange (SCE) assay is a sensitive biomarker to detect DNA damage (Alptekin *et al*. 2006). It represents the symmetric interchange between homologous *loci* of replication products (Wolff 1982). SCE occur without either loss of DNA or changes in the chromosomal morphology, and it is possible to detect them in metaphase. The assay is based on the incorporation of the thymidine DNA base analog 5-bromo 2´-deoxyuridine (BrdU) into the DNA of cells that replicated twice (Latt 1979, Latt *et al*. 1981). In addition to SCE analysis, the BrdU differential staining technique can be used to assess the effects of pesticides in cell replication through the cell proliferation kinetics (CPK; Gómez-Arroyo *et al*. 2000a).

Organophosphorus pesticides are common compounds involved in poisoning. They act by inhibiting the hydrolysis reaction performed by acetylcholinesterase, an enzyme that is essential for the central nervous system function in insects and humans. Such inhibition leads to accumulation of the neurotransmitter acetylcholine, causing interruption of nervous impulses in the synapses (Eyer 2003). The routes of exposure to insecticides are absorption through skin, inhalation and ingestion of contaminated water or vegetables.

Organophosphorus insecticides are widely used in Mexico (Gómez-Arroyo *et al*. 2000b, González *et al*. 2002, Martínez-Valenzuela *et al*. 2009). Some of these insecticides, such as gusathion or azinphos methyl, are classified by the World Health Organization (WHO 2010) as IB class ("highly hazardous") and by the PAN International List of Highly Hazardous Pesticides (2014) as Groups 1 (acute toxicity) and 3 (environmental toxicity). These compounds can be metabolized by plants. Metabolites or their residues can then remain stored in edible plants and may be ingested by humans, where the metabolic machinery can transform them into more toxic products (Plewa *et al*. 1988, 1993, Cortés-Eslava *et al*. 2001, Gómez-Arroyo *et al*. 2007).

The aim of the present study was to evaluate the capacity of broad bean (*Vicia faba*) S10 fraction to metabolize the organophosphorus insecticide azinphos methyl and the influence of such metabolites in the induction of reverse mutation in *Salmonella typhimurium* TA98 and TA100 strains and in the SCE frequency in human lymphocyte cultures.

MATERIALS AND METHODS

Chemicals

The chemicals used were the following: 4-nitro-*o*-phenylenediamine (NOP)-CAS registry number N-99-56-9, 2-aminofluorene (2AF)-CAS number 108-45-1, bromodeoxy uridine (BrdU)-CAS number 59-14-3. The latter was purchased from Sigma Chemical Co., St. Louis, MO, USA. Gusathion or azinphos methyl (O,O-dimethyl-S- [4-oxo-1, 2, 3-benzotriazin-3(4H)-yl] methyl phosphorodithioate (CAS number 86-50-0, was bought from Bayer de México), guaiacol (CAS number 90-05-1, was purchased from Reasol of México), hydrogen peroxide (CAS number 7722-84-1 was purchased from J.T. Baker of México). The other reagents used were of analytical grade. Roswell Park Memorial Institute (RPMI) medium 1640 with L-glutamine and phytohemagglutinin was purchased from Gibco.

Preparation of the bacterial suspension

The *Salmonella* tester strains TA98 and TA100 were maintained frozen at –80 ºC as recommended by Maron and Ames (1983). Cells were incubated in Luria broth (LB) medium for proliferation, and master plates were prepared. Strain genetic markers were tested in all mutagenic experiments according to the method of Zeiger *et al*. (1981). The range of spontaneous number of revertant colonies per plate in TA98 was from 27 to 49 and in TA100 was from 148 to 173.

Mutagenic activity of azinphos methyl was determined through the S10 *Vicia faba* fraction (plant metabolism) using NOP and 2AF as positive controls. Bacteria were grown overnight in Oxoid Nutrient Broth No. 2 at 37 ºC with shaking in darkness for 16 h, washed in 100 mM of potassium phosphate buffer pH 7, and the bacterial suspension title was determined spectrophotometrically and adjusted to 1×10^{10} colony-forming units/mL in phosphate buffer. The experiments were performed under

yellow light in order to avoid photo-oxidation of mutagens (Nishi and Nishioka 1982).

Vicia faba S10 mix preparation

Vicia faba (var. minor) seeds were germinated between two cotton layers soaked in tap water. When the primary roots reached a length of 3-5 cm were rinsed with distilled water and cut at approximately 2 cm from the primary tips. The roots were then macerated and homogenized at 4 ºC in 0.1 M sodium-phosphate buffer, pH 7.4. The ratio of the buffer solution to the fresh weight of the root cuttings in grams was 1:1 v/w (Takehisa *et al*. 1988, Gómez-Arroyo *et al*. 2000a). The homogenized roots were centrifuged for 15 min at 10 000 g at 4 ºC. The supernatant was sterilized by filtration using Millipore membranes (0.45 μm pore size). The total protein concentration in these extracts was determined using the Bio-Rad method (Bradford 1976). Protein concentration was fairly constant from experiment to experiment, with values from 3.34 to 3.52.

The metabolic activation system with the S10 mixture was prepared from the microsomal S10 fraction at a 1:9 ratio (v/v) with the following compounds: 8 mM $MgCl_2$, 3.3 mM KCl, 5 mM glucose-6 phosphate, 4 mM NADP and NAD, and 0.1 M Na_2HPO_4-NaH_2PO_4 buffer at pH 7.4.

Mutagenicity assays

In test tubes, increasing concentrations of the insecticide azinphos methyl (20, 40, 80, 100 and 200 μg/μL) and 100 μL of the bacterial suspension of TA98 or TA100 were added to the direct treatments (without plant metabolism). In the indirect treatments (with metabolic activation), 500 μL of S10 mix were incubated with the corresponding insecticide concentration plus 100 μL of bacterial suspension. In both cases (direct and indirect treatments) phosphate buffer was added making a final volume of 2.5 mL. The tubes were shaken (12 rpm) for 1.5 h at 28 ºC in darkness. Finally triplicate 0.25 mL aliquots of incubation mix were added to 2 mL of molten top agar supplemented with 0.5 M histidine/biotin, vortexed, poured into Vogel-Bonner (V-B) minimal medium agar plates and incubated for 48 h at 37 ºC (Maron and Ames 1983). *Salmonella* revertant *his*$^+$ colonies were scored with a digital counter (New Brunswick Scientific C-110). Three parallel plates were made for each concentration tested, and each experiment was repeated three times.

The insecticide concentrations were based on preliminary assays as the best for obtaining a significant activation without affecting bacterial viability, which was determined by observing the background lawn of bacterial growth (De Flora *et al*. 1992).

Peroxidase activity determination

To evaluate peroxidase activity, we measured the oxidation of guaiacol to tetraguaiacol by observing the change in absorbance at 470 nm (Chance and Maehly 1955). Peroxidase activity was detected at 30-sec intervals over a 5-min time period. The experiments were repeated three times for each sample as described by Gentile and Gentile (1991). The peroxidase activity rate was calculated as described by Gichner *et al*. (1994), and the reaction rate of peroxidase was obtained in nmol of tetraguaiacol/mg of protein/min.

For each treated group the following equation was applied:

$$\text{Peroxidase reaction rate} = \frac{\left[\left(\frac{A_{470}}{\varepsilon_{470} \times l}\right) \nu / p\right]}{t_{min}}$$

where A_{470} is the absorbance at 470 nmol, ε_{470} is the extinction coefficient of tetraguaiacol (26.6 mM/cm), l is the path length of the cuvette**,** n is the volume of the reaction in liters, p is the protein content in mg and t_{min} is the time in minutes. The peroxidase activity of the cultures is expressed as a percentage of the initial value.

Treatments with azinphos methyl applied directly and using *in vitro* promutagen activation by *Vicia faba* S10 mix in human lymphocyte cultures

Lymphocytes cultured for 48 h were exposed to azinphos methyl 2, 4, 8, 10, 20, 30 and 40 μg/mL (chosen in preliminary experiments) for 4 h (cultures were stationary for the first two hours and shaking for the second two hours) in the dark at 37 ºC, with and without metabolic activation. After treatment, the cells were rinsed twice in 0.9 % sodium chloride and incubated for 24 h in an RPMI medium containing BrdU at a final concentration of 5 μg/mL. Colchicine (0.1 mL, 5×10^{-6} M) was added 70 h after the start of the culture.

The metabolic activation system with the S10 mixture was prepared from the microsomal S10 fraction at a 1:9 ratio (v/v) with the following compounds: 8 mM $MgCl_2$, 3.3 M KCl, 5 mM glucose-6 phosphate, 4 mM NADP and NAD, and 0.1 M Na_2HPO_4-NaH_2PO_4 buffer at pH 7.4. The 48 h cultures were incubated for 4 h at 37 ºC with 500 μL of the activation system and the different concentrations of the insecticide. Ethanol 0.1 M was used as a positive control. Ethanol is a proved promutagen in *Vicia faba*

and increases the SCE frequency (Takehisa *et al*. 1988, Gómez-Arroyo *et al*. 1995, 2000a, Calderón-Segura *et al*. 1999, Flores-Maya *et al*. 2005).

Metaphase cells were harvested by centrifugation, treated with 0.075 M of KCl for 20 min, and fixed in methanol-acetic acid (3:1). The slides were prepared by the air-drying method, dropping the cell suspension onto the wet slide, and stained using the fluorescence-plus-Giemsa technique (Perry and Wolff 1974). In addition to the study of SCE, a BrdU differential staining technique was used to assay the effect of azinphos methyl on cell replication. For evaluation of cytokinetics, the proportion of first (M1), second (M2), and third (M3) metaphases were obtained from 100 consecutive mitoses of each treatment, and the replication index (RI) was calculated as follows: RI = 1M1 + 2M2 + 3M3/100 (Lamberti *et al*. 1983); the mitotic index was also determined. The slides were blinded to avoid bias.

Statistical analysis

Differences were tested among the treated groups within the experimental series through the analysis of variance. When a significant F value ($p < 0.0001$) was found, each treated group and its corresponding negative control were tested for significance using a Student-Newman-Keuls multiple comparison test at $p < 0.001$. A *chi* squared (χ^2) test was used for RI and MI. The parts of the decomposed χ^2 were used to compare the values of M1, M2, and M3.

RESULTS

Table I shows the data from the mutagenic evaluation of the insecticide applied to strains TA98 and TA100, either directly or following *Vicia faba* S10 mix. Azinphos methyl applied directly was toxic for the TA98 strain, which was demonstrated by the total disappearance of the background lawn. This toxicity decreased in the presence of plant metabolism, giving the appearance of the background and of revertant colonies in the TA98 strain at several insecticide concentrations up to 200 µg/µL. In the TA100 strain, the insecticide directly applied was less toxic and its plant metabolites also induced mutagenicity. In all cases, the behavior of the positive and negative controls was in agreement with the values described previously.

In the samples treated with azinphos methyl, peroxidase activity increased after the treatment,

TABLE I. MUTAGENIC EVALUATION OF AZINPHOS METHYL WITHOUT AND WITH PLANT[a] METABOLISM IN *S. typhimurium* TA98 AND TA100 STRAINS

	[b]Revertants/plate			
	TA98		TA100	
Negative controls	–S10	+S10	–S10	+S10
[b] Mean	27 ± 4	49 ± 4	148 ±14	173 ± 11
Positive controls				
[c] NOP	659 ±74*	1685 ±102*	274 ± 9*	413 ± 18*
[d] 2-AF	185 ±17*	1642 ± 30*	237 ± 2	1517 ± 21*
Treatments (µg/µL)				
Azinphos methyl				
20	19 ± 5†	122 ± 3*	79 ± 5	144 ± 6
40	18 ± 4†	226 ± 13*	167 ± 4	193 ± 3*
80	19 ± 3†	494 ± 24*	83 ±13	314 ± 2*
100	17 ± 2†	492 ± 10*	165 ± 2	312 ± 21*
200	18 ± 3†	9 ± 2†	114 ± 8	409 ± 16*

* Significant differences between the control group and each treated group were obtained by analysis of variance for TA98, F = 102.499 and for TA100, F = 127.64 F value is $p < 0.0001$, and therefore the Newman- Keuls multiple comparison test was applied $p < 0.001$.

[a] *Vicia faba* S10 mix.

[b] Mean of revertants obtained in three independent assays ± S.E.

[c] 4-nitro-*o*-phenylenediamine (100 µg/µL).

[d] 2-aminofluorene (20 µg/µL)

† Values lower to the negative control produced by toxicity

whereas in samples treated with NOP and 2AF, both values remained similar (**Table II**).

TABLE II. PEROXIDASE ACTIVITY IN S10 *Vicia faba* FRACTION BEFORE AND AFTER 48 HOUR TREATMENT WITH NOP, 2-AF AND THE INSECTICIDE AZINPHOS METHYL

	Peroxidase activity[a] (nM of tetraguaiacol/min/μg of protein)	
	Before treatment	After treatment
S10 mix	0.065 ± 0.08	0.056 ± 0.04
Treatments (μg/μL)		
NOP[b]		
100	0.230 ± 0.01 N.S.	0.228 ± 0.06 N.S.
2AF[C]		
20	0.225 ± 0.03 N.S.	0.232 ± 0.09 N.S.
Azinphos methyl		
5	0.061 ± 0.08 N.S.	0.126 ± 0.09*
40	0.054 ± 0.07 N.S.	0.178 ± 0.06*
100	0.061 ± 0.04 N.S.	0.149 ± 0.09*

N.S.: No significant differences between the control and each treated group were obtained by analysis of variance.
* Significant differences between the control and each treated group were obtained by analysis of variance, F = 60.567, F value is $p < 0.0001$, and therefore the Newman- Keuls multiple comparison test was applied $p < 0.001$.
[a] Mean of three assays ± S.E.
[b] 4-nitro-*o*-phenylenediamine.
[c] 2-aminofluorene.

Table III shows that there is no difference between the SCE frequencies and those of the negative control when azinphos methyl is applied directly. On the other hand, when S10 mix was added, a concentration-response relationship was observed starting at 8 μg/mL and a positive response was obtained ($p < 0.001$). The cell kinetics and the replication index are also listed in **Table III**. No significant differences were found with 2 and 4 μg/mL. However, starting at 8 μg/mL M1 increased and M3 significantly decreased, RI and MI diminished as the concentration was increased up to 400 μg/L in which mitotic inhibition was observed. As expected, significant differences were observed in SCE frequency, cell kinetics and RI with the positive control ethanol. No significant differences were found in the MI.

DISCUSSION

Azinphos methyl is not mutagenic in *Schizosaccharomyces pombe* (Degraeve *et al*. 1980), however Gilot-Delhalle *et al*. (1983) found a positive response in the same yeast and Bianchi *et al*. (1994) in *Saccharomyces cerevisiae*. It does form DNA adducts in calf thymus in the presence of the S9 fraction (Shah *et al*. 1997). It does not induce SCE in human lymphocyte cultures (Gómez-Arroyo *et al*. 1987) while in plants it causes SCE in *Vicia faba* (Gómez-Arroyo *et al*. 1988).

The results obtained in the Ames assay in which several *Salmonella typhimurium* strains were exposed to azinphos methyl without and with S9 metabolic activation have been contradictory because in some studies the response is negative (Simmon *et al*. 1976, Carere *et al*. 1978, Garret *et al*. 1986), in another it is weak positive (Zeiger *et al*. 1987), and when the plant cell/microbe coincubation assay with tobacco cells is applied, a positive response is observed (Gómez-Arroyo *et al*. 2007).

In the present study, the direct application of azinphos methyl did not induce mutagenesis but prevented background lawn growth in all concentrations. According to De Flora *et al*. (1992), toxicity to bacteria is indicated by the disappearance of the background lawn.

It is important to choose carefully the source for plant homogenates, since the presence and activity of an enzymatic system may vary in different plant tissues and at different developmental stages (Callen 1982). In this study we investigated the activation of azinphos methyl by cell-free extracts of *Vicia faba* roots, because not all the plant species can activate the same promutagen. Azinphos methyl was previously tested by Gómez-Arroyo *et al*. (2007) with the plant cell/microbe coincubation assay using *Salmonella typhimurium* TA98 and TA100 strains as a the mutagenic indicator organism and *Nicotiana tabacum* cells as a promutagen activator, and by Cortés-Eslava *et al*. (2014) with *Coriandrum sativum* cells. In both cases they found that the peroxidase activity decreases in treatments when the insecticide is metabolized. However, our study, in which a remarkable increment of peroxidase enzymes by azinphos methyl was observed, is in disagreement with the results mentioned above. For instance, in *Vicia faba* S10 fraction other mechanisms could be involved, possibly through the formation of DNA adducts due to the action of alkylating groups. In this way, Shah *et al*. (1997) demonstrated that azinphos methyl form DNA adducts in calf thymus in the presence of the S9 fraction. Stakes *et al*. (1995) found similar results in urine of applicators, were the short-term exposure to the pesticide was validated by the presence of dimethyl thiophosphate, a metabolite of azinphos methyl.

TABLE III. SISTER CHROMATID EXCHANGES AND EFFECTS ON CELL KINETICS (M1, M2 AND M3 CELLS), REPLICATION INDEX (RI) AND MITOTIC INDEX (MI) IN HUMAN LYMPHOCYTE CULTURES INDUCED BY AZINPHOS METHYL WITHOUT AND WITH *Vicia faba* METABOLIC FRACTION (S10)

	SCE X[a] ± S.E.	M1	M2	M3	% RI[b]	MI
Negative controls						
Lymphocytes	4.82 ± 0.21	32	34	21	2.01	3.75
S10	5.28 ± 0.20	28	33	39	2.11	3.70
Ethanol [c] - S10	4.56 ± 0.23	33	38	29	1.96	3.15
Positive control						
Ethanol[c] + S10	9.12 ± 0.35*	17*	38	45	2.28	3.35
Treatments						
Azinphos methyl[e] –S10						
2	4.50 ± 0.20	25	33	42	2.17	3.50
4	4.80 ± 0.18	27	39	34	2.13	3.12
8	4.92 ± 0.28	25	39	36	2.11	3.62
10	4.88 ± 0.32	29	40	31	2.02	3.35
20	4.26 ± 0.45	31	43	26	1.95	3.43
30	4.62 ± 0.57	34	41	25	1.91	3.24
40	4.86 ± 0.26	29	43	31	2.08	3.14
Azinphos methyl[e] + S10						
2	5.64 ± 0.23	36	28	36	2.00	3.10
4	5.16 ± 0.17	43	33	22**	1.79	2.55
8	7.16 ± 0.31*	46**	30	24**	1.78	1.95
10	7.18 ± 0.31*	49**	30	21**	1.70**	1.95
20	8.70 ± 0.26*	51**	28**	21**	1.70**	1.45**
30	9.04 ± 0.38*	57**	25**	18**	1.61**	1.30**
40	f	f	f	f	f	f

[a] Mean of 50 metaphase cells in two independent assays ± S. E.
[b] Replication index, n = 200 consecutive metaphases.
[c] Ethanol concentration (0.1 M).
[e] Azinphos methyl to µg/mL.
[f] Mitotic inhibition (stimulated cells were not observed).
* Significant differences among controls and each treated group were obtained by analysis of variance F = 31.102 F value is < 0.0001, and therefore the Newman - Keuls multiple comparison test was applied $p < 0.001$.
** Significant with X^2, $p < 0.05$.

Plewa *et al*. (1984) suggested that some chemicals may be activated in plants by similar enzymatic systems as those of animals. Takehisa *et al*. (1988) found that ethanol induced SCE after *Vicia* S10 activation as well as after rat-liver S9 activation. The strength in activating ethanol was stronger with Vicia S10 than with rat-liver S9. For this reason the activation capacity specific for *Vicia faba* could be also found for azinphos methyl.

The Vicia faba products obtained from the in vitro promutagen activation of azinphos methyl applied to *Salmonella typhimurium* and to lymphocyte cultures were capable of increasing the reverse mutations in TA98 and TA100 strains and SCE frequency, respectively, which means that this compound acted indirectly. However, toxicity diminished with plant metabolism, possibly indicating that some detoxification mechanisms were involved. The same detoxification was observed with the thiocarbamate herbicides molinate and butylate in the presence of the S10 fraction (Calderón-Segura *et al*. 1999).

CONCLUSIONS

The products obtained from the *in vitro Vicia faba* promutagen activation of azinphos methyl applied to *Salmonella typhimurium* were capable of increasing the reverse mutations in TA98 and TA100 strains. These results indicate that the mechanism to induce mutations by the insecticide azinphos methyl was frameshift mutation (in TA98 strain) as well as pair base substitution (in TA100 strain). However, toxicity diminished with plant metabolism, suggesting that some detoxification mechanisms were involved. Also, the SCE frequency in human lymphocyte

cultures was significant and a dose-response relationship was observed, which means that gusathion or azinphos methyl acted indirectly in both biological systems.

ACKNOWLEDGMENTS

The authors would like to thank the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPITT-DGAPA), Universidad Nacional Autónoma de México (UNAM) for financial support through the project IN227305. The authors thank Ana Rosa Flores Márquez for her technical assistance and Claudio Amescua for his scientific editing.

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