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Cytotoxicity and Mutagenicity of Four Insect Pheromones in CHO-K1 Cells

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Integrated Pest Management (IPM) procedures include a combination of biological, cultural, physical, mechanical and chemical tools in such a way that minimizes economic, health, and environmental risks and increase the sustainability of farming systems by reducing the use of conventional toxic pesticides (Dent, 1993). IPM includes soil preparation, planting practices, pest trapping, use of environmentally friendly chemicals, natural enemies or cultural methods to maintain pests at acceptable levels, avoidance many of the important negative effects of conventional pesticide use, like the mortality of beneficial insect species and selection of resistant plant strains. Environmental-friendly insecticides include plant derived allelochemicals, mycoinsecticides, entomopathogenic nematodes, insect growth regulators and natural or synthesized insect sex pheromones. These compounds can effective control on insect pest populations. The mode of action of these substances is based on insect biology and physiology, thus providing greater target pest specificity, rapid degradation and low persistence and toxicity in the environment.

Today, the application of insect sex pheromones in insect pest control is considered one of the basic techniques of IPM programs. These compounds modify sex-coupling behavior, disrupting effective insect mating. A sufficiently high concentration of these compounds in the air, blankets the trails of female insects, confusing the males, who are unable to trace the pheromone source and thereby diminishing insect propagation. Many insect pheromones marketed as insect sex attractants, are currently used against specific insect pest in IPM programs. Manufacturers claim that, due to the natural origin of these compounds, they are non toxic agents. As the matter of fact, animal trials using gossyplure - the natural pheromone of pink bollworm *Pectinophora gossypiella* - and virelure - the natural pheromone of the artichoke plume moth *Platypillia candidactylia* - showed no toxic effects up to 5 g/kg (p.o. male rats), 2 g/kg (dermal exposure, rabbits) and 5 g/L (air exposure, male rats) figures that would indicate that these substances can be safely managed. We have shown that the lack of basal cytotoxic effects of these compounds could be attributable to the strong binding to serum proteins (Bayoumi et al, 2002).

In the present study we studied the *in vitro* cytotoxicity effects of four lepidopte---

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ran pheromones: *trans*-8, *trans*-10 dodecadienyl acetate (8,10-DDDA) natural pheromone from *Rhyacionia rigidana*, *trans*-8, *trans*-10 dodecadienol (8,10-DDDOL) natural pheromone from *Laspeyresia pomonella*, *cis*-7 tetradecen-1-yl acetate (7-TDDA) and *trans*-7, *cis*-9 dodecadienyl acetate (7,9-DDDA) natural pheromones from *Lobesia botrana* on CHO-K1 cells, as well as their mutagenicity on *Salmonella typhimurium* reversion assay.

MATERIALS AND METHODS

Reagent grade chemicals and cell culture components were used: culture medium HAM F-12, penicillin/streptomycin solution (10,000 U/ml penicillin, 10 mg/ml streptomycin in 0.9 % NaCl), trypsin/EDTA solution (1 %), HEPES, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) 98 %, bovine serum albumin (BSA) 96 %, NADP 97 %, glucose-6-phosphate 98 % and dimethyl sulfoxide (DMSO) 99.5 %, were obtained from Sigma Chemical Co. (ST. Louis, MO. USA). Fetal calf serum (FCS) was obtained from Boehringer Ingelheim (Germany). The insect pheromones *trans*-8, *trans*-10 dodecadienyl acetate (E,E-8,10-DDDA pheromone from *Rhyacionia rigidana*) 95 %, *trans*-8, *trans*-10 dodecadienol (E,E-8,10-DDDOL pheromone from *Laspeyresia pomonella*) 95 %, *cis*-7 tetradecen-1-yl acetate (Z-7-TDDA) 96 % and *trans*-7, *cis*-9 dodecadienyl acetate (E,Z-7,9-DDDA pheromone from *Lobesia botrana*) 95 %, were obtained from Sigma Chemical Co., (St. Louis, MO. USA). To prepare the primary stock solutions, each compound was completely dissolved in filter-sterilized DMSO, whereafter the stock solutions were dissolved in the culture media to reach the desired dilutions. DMSO in the cultures never exceed of 0.2 % (v/v).

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL 61). Ten thousand cells/cm^2 were plated in monolayers in 22 cm^2 polystyrene tissue culture dishes with HAM F12 medium (Sigma Chemical Co.) supplemented with 25 mM HEPES buffer (pH 7.4) and 10% heat-inactivated FCS and gentamicin (30 μg/mL). Cells were counted on an improved Neubauer hemocytometer and cell viability was determined by exclusion intake of Trypan Blue dye. Cells were allowed to reach 65 % confluence (day 3, mid log-phase) and then pulsed with different concentrations of the insect pheromones. They were subsequently washed and the medium was replaced after 24, 48 and 72 hr, and cell viability was determined by the method mentioned below.

The MTT assay method described by Carmichael et al, (1987 a,b) was used to estimate insect pheromone cytotoxicity in CHO-K1 cells. Cells were allowed to attain 65 % confluence and then pulsed with different concentrations of the insecticides tested. Cultures were exposed to the agents for 24, 48 and 72 hr in absence of FCS or presence of 1 % BSA. After incubation, cells were transferred to a fresh medium containing 50 μl MTT at a concentration of 50 mg/ml, incubated during three hours at 37 °C with the cells. The non-metabolized reagent was eliminated and 100 μl DMSO was added. The medium was then removed (4

min at 37 °C and complete darkness) and the absorbance was measured at dual wavelength 570/690 nm with Multiskan MCC/340P spectrophotometer.

Salmonella typhimurium TA 98, TA 100 and TA 102 strains were provided by Bruce Ames (University of California, Bekerley). Reagents and positive controls (2-aminoanthracene 96 %, 2-nitrofluorene 98 % and methylmethane-sulfonate 96 %) were obtained from Aldrich Chemical Company (Milwaukee, WI). Tester strains were exposed to four pheromones in according to Maron and Ames (1983). Three concentrations of each pheromone along with appropriate vehicle and positive controls were plated, i.e. 1, 50 and 100 μg with testers strains in presence and absence of rat liver S9-activation. Rat liver submitochondrial S9-mix was freshly prepared as described by Venitt et al. (1984). Four percent of S9-mix was used in this study. The final mixture contained 1 M KCl, 0.25 M $MgCl_2$, 0.2 M glucose-6-phosphate, 0.04 M NADP, 0.2 M $NaHPO_4$, deionized water and the S9 fraction obtained from Iffa Credo (Charles River Co. Les Oncins, France).

RESULTS AND DISCUSSION

Lepidopteran pheromones consist of unbranched aliphatic chains with alcohol, aldehyde or acetate functions at the end of the molecule which interact with male receptors and disrupt effective mating. The acute toxicity of these substances for non-target organisms, i.e. reptilians, birds or mammals, has been considered to be non-relevant under field use conditions, and pheromone use is accepted as a good agricultural practice.

Cytotoxicity values derived from dose-response curves show that the toxic effect of each compound was time-dependent. Cytotoxicity values in Table 1, and those illustrated in Fig. 1, show that the pheromones were significantly less toxic in the absence of FCS compared to those cultures which were incubated with 1 % BSA. After 24 and 48 h, and according to the cytotoxicity values (IC_{20}, IC_{50} and IC_{80}) the pheromones ranked as follow: Z-7-TDDA > 8, 10-DDDA > 7, 9-DDDA > 8, 10- DDDOL.

Despite the significant cytotoxicity of these compounds, there are several reasons to explain their potential safety. Similarly to other pheromones (Bayoumi et al., 2002), the presence of proteins in the culture medium can change cytotoxicity parameters due to unspecific binding or hydrolytic reactions. Due to their long hydrophobic aliphatic chain, resembling normobiotic fatty acids, these compounds can be carried and/or distributed by serum albumin, reducing cellular bioavailability and undesirable effects. Unfortunately, no toxicokinetic studies have been carried out in experimental animals to date, but binding to serum proteins after absorption may be one of the reasons for their low toxicity after acute or chronic exposure (Vogt et al, 1985; Blatter et al, 1990; Bayoumi et al, 2002). On the other hand, the conditions of use of these compounds make results in very limited exposure to non-target animals. Accidental spillage of insect pheromones into aquatic environments is unlikely, however, due to the particular

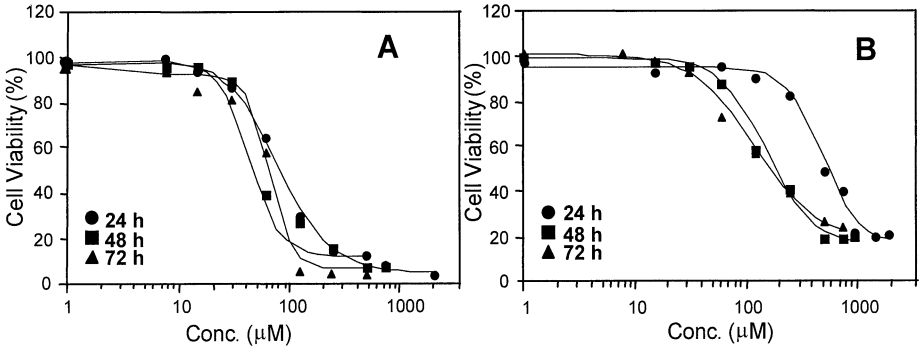

Figure 1. Cytotoxicity of 7,9-dodecadienyl-acetate (7,9-DDDA) as one of the tested insect sex pheromones after different periods of incubation with CHO-K1 cells in serum free medium (A) and with medium containing 1 % BSA (B) using MTT assay.

way such substances are handled. Indeed, pheromones are used to bait traps of diverse designs, often consisting of open-ended plastic hollows or plastic bags that emit the volatile product through both sides of the walls, respectively, thus reducing liquid spillage or undesirable contamination to these compounds.

These substances seem to be easily metabolized by a variety of enzymes, i.e. carboxylesterases (Vogt et al, 1985) and aldehyde dehydrogenases (Blatter et al, 1990). Similarly, Bayoumi et al. (2002) found that the insect pheromones ZZ/ZE-7, 11-hexadecadienyl acetate, 7-11-hexadecenal, Z-9-hexadecenal and Z-11-hexadecen-1-ol exhibited significantly lower cytotoxicity to CHO-K1 as the effect of their binding to protein and their metabolism by S9 fraction which offers oxidative mechanisms prompt to produce non cytotoxic metabolites.

The mutagenic potential of insect sex pheromones was assessed using *S. typhimurium* TA98, TA100 and TA102 strains alone, or after previous treatment with rat submitochondrial S9 fraction (Table 2). Comparison between of the revertant colony numbers obtained with the tested substances and that recorded by positive control in each tested strain, showed that the tested agents did not exhibited significant mutagenic potential either in absence or in presence of S9-mix at the three tested concentrations, i.e. 1, 50, 100 and/or 500 μg/dish.

No evidence of genotoxicity was found in other long-aliphatic chain acids or alcohols. Lanigan et al. (2001) showed that octyldodecyl stearoyl stearate lacked *in vitro* mutagenic effect using both a mammalian test system or in the Ames test system, with or without metabolic activation. Similar negative results were obtained by Iglesias et al., (2002) were obtained with behenyl alcohol [a saturated long-chain fatty alcohol (C22:0)] conducting gene mutation and chromosome aberration assays. Furthermore, Gamez et al., (2002) found that the agent D-003

Table 1. Cytotoxicity values, (IC_{50}) of the tested insect pheromones after exposing to CHO-K1 cell line during different periods using the mitochondrial function assay (MTT) in absence or presence of proteins.

Compound	IC_{50} (μM)					
	Serum free medium			1 % BSA		
	24 h	48 h	72 h	24 h	48 h	72 h
7, 9-DDDA	95.5 ± 6.6	79.4 ± 10.1	54.9 ± 11.7*	489.8 ± 65.0$^{\$}$	208.9 ±15.2$^{\$}$	186.2 ± 11.6$^{\$\$}$
8, 10-DDDA	60.3 ± 12.3	44.7 ± 13.6**	54.9 ± 10.1	371.5 ± 32.9$^{\$\$}$	338.8 ± 8.7$^{\$}$	218.8 ± 11.9$^{\#\#\ \$\$}$
8, 10-DDDOL	229.1 ± 12.0	177.8 ± 9.6**	145.6 ± 5.6**	1500.0 ± 11.5$^{\$\$\$}$	1479.1 ±15.2$^{\#\ \$\$\$}$	1732.1 ± 19.6**
Z-7-TDDA	22.9 ± 4.10	22.4 ± 3.7	18.2 ± 2.9*	323.6 ± 7.2$^{\$\$}$	251.1 ± 23.1$^{\#\ \$\$\$}$	208.9 ± 11.9$^{\#\#\ \$\$}$

***/###/$$$: Highly significant ($P \geq 0.001$), **/##/$$: Moderately significant ($P \geq 0.01$), */#/$: Significant ($P \geq 0.05$)

*/#: Significant difference between the exposed times of 24, 48 and 72 h inside each assay.

$: Significant difference between the exposed times of 24, 48 and 72 h between the two assays.

Table 2. Mutagenic potentials of insect sex pheromones against *S. typhimurium* TA98, TA100 and TA102 in the absence and presence of S9-fraction.

Compound	Conc. (μg/Dish)	Revertant colony number					
		TA98		TA100		TA102	
		S9(-) mean ± SD	S9(+) mean ± SD	S9(-) mean ± SD	S9(+) mean ± SD	S9(-) mean ± SD	S9(+) mean ± SD
Negative control		30 ± 3	20 ± 5	132 ± 20	191 ± 38	282 ± 37	274 ± 26
7,9-DDDA	1	33 ± 1	27 ± 3	168 ± 2	241 ± 12	167 ± 8	353 ± 16
	50	35 ± 1	26 ± 5	171 ± 20	264 ± 15	244 ± 17	347 ± 47
	100	24 ± 0	27 ± 4	152 ± 21	230 ± 0	337 ± 16	395 ± 112
8,10-DDDA	1	27 ± 1	24 ± 7	129 ± 1	213 ± 0	206 ± 46	374 ± 23
	50	30 ± 1	23 ± 5	111 ± 12	186 ± 68	267 ± 14	344 ± 8
	100	32 ± 2	21 ± 0	113 ± 4	197 ± 60	245 ± 8	340 ± 14
DDDOL	50	31 ± 13	39 ± 25	140 ± 23	164 ± 21	160 ± 27	385 ± 0
	100	36 ± 17	27 ± 23	121 ± 18	141 ± 28	168 ± 11	322 ± 7
	500	37 ± 15	33 ± 8	132 ± 21	151 ± 12	157 ± 4	395 ± 30
TDDA	50	16 ± 11	30 ± 3	151 ± 6	159 ± 28	199 ± 15	522 ± 6
	100	28 ± 8	21 ± 4	130 ± 1	157 ± 21	175 ± 8	548 ± 8
	500	27 ± 2	31 ± 0	137 ± 8	91 ± 6	181 ± 26	441 ± 22
Positive controls a & b		2780 ± 123(a)	3550 ± 71(b)	2898 ± 141(a)	2683 ± 424(b)	2950 ± 71(a)	967 ± 261(b)

a 2-Nitrofluorene (0.2 μg/plate, TA98), methylmethane sulfonate (1.5 μl/plate, TA100, TA102).

b 2-aminoanthracene (4 μg/plate, TA98, TA98 and TA102 + S9 mix).

(a mixture of very long chain aliphatic acid containing octacosanoic acid as the major component) did not increase the frequency of reverse mutations in the Ames test in both alternatives with or without S9 mixture.

Lepidopteran pheromones produced significant cytotoxicity in CHO-K1 cells. This cytotoxicity was mitigated with 1 % BSA. Furthermore, no mutagenic effect was found using the Salmonella-reversion test with and without submitochondrial pre-incubation.

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