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Pesticide Induced Toxicity and Stress Response in Bacterial Cells

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Synthetic pesticides such as fungicides and herbicides are among the most widely used chemicals in agriculture (Hayo 1996). By their nature, some pesticides may cause some risk, to humans, animals, and the environment because they are designed to adversely affect living organisms.

To assess the impact of pesticides on public health and the ecosystem, biological test systems have been developed and applied to complement chemical and physical testing (Amadeo et al. 2002). The use of biosensing systems has primarily focused on detecting the genotoxicity of pesticides (Ruiz and Marzin 1997). However, pesticides can also affect other cellular mechanisms and components including proteins and the cellular membrane.

The detection methods used for such toxic chemicals entered a new era upon the introduction of recombinant bioluminescent bacterial cells, which contained a fusion of a stress response promoter and a bioluminescence reporter gene for detecting the several kinds of cellular stress and toxicities induced by toxic chemicals including pesticides. The stress promoters regulate the synthesis of many different stress proteins on the transcriptional level, which helps the cells adjust themselves to a new environment when they are exposed to toxic or hazardous situations. A wide range of stress genes is known to exist in prokaryotic cells (Bulich 1952; Rupp 1996) including the *recA* gene, in which *recA* is a gene expressed rapidly under DNA damaging conditions in bacteria. Fusion of stress promoters, such as the *recA, katG, fabA, grpE, and sodA* promoters, with the *lux* genes results in new cellular biosensing strains, which emit light when stressed, and many different recombinant bioluminescent bacteria have been constructed (Belkin et al. 1996; Choi and Gu 2001; Lee and Gu 2003; Van Dyk et al. 1995; Vollmer et al. 1997). These recombinant bioluminescent biosensing strains have been used to measure the toxicity of many different chemicals based on their modes of toxic actions. For example, a recombinant *E. coli* DPD2794 containing a *recA* promoter region fused to *luxCDABE* originating from *Vibrio fischeri* was used to detect DNA damage by toxicants including mutagens, for example, mitomycin C (MMC), benzo[a]pyrene, some endocrine disrupting chemicals (EDCs), and γ-rays (Gu et al. 2002; Min et al. 1999; Vollmer et al. 1997). As well, other strains, DPD2540, with the *fabA* promoter, DPD2511, with the *katG*

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promoter, and TV1061, with the *grpE* promoter, were used to detect specific toxicant types, *i.e.*, membrane, oxidative and protein damaging agents (Belkin et al 1996; Choi and Gu 2001; Gu et al. 2002; Min et al. 1999; Van Dyk et al. 1995).

In this study, these four recombinant bioluminescent bacteria were used to classify the specific stress caused to bacterial cells by the addition of pesticides. The classification of the seven pesticides - fungicides ziram, ethylene dibromide, and benomyl, insecticides fenvarelate, methidathion, and methoxychlor, and the herbicide glyphosate - according to their modes of toxic actions was studied using the four strains. Finally, GC2, a strain harboring a plasmid with the *lac* promoter fused to the *luxCDABE* operon from *Xenorhabdus luminescens* (Marincs and White 1994), was used to detect the general cellular toxicity caused by pesticides. Therefore, using these five recombinant bioluminescent bacteria, each pesticide was classified according to its potential toxicity and the specific type of stress induced.

MATERIALS AND METHODS

The recombinant bioluminescent bacteria, *Escherichia coli* strains DPD2794 (*recA::luxCDABE*) (Vollmer et al. 1997), DPD2540 (*fabA::luxCDABE*) (Choi and Gu, 2000), DPD2511 (*katG::luxCDABE*) (Belkin et al. 1996), and TV1061 (*grpE::luxCDABE*) (Van Dyk et al. 1995) were constructed at DuPont Co., USA and employed in this study. Each recombinant strain had a specific stress promoter fused to the *luxCDABE* operon originating from *Vibrio fischeri*. A fifth recombinant bioluminescent bacteria, GC2 (*lac::luxCDABE*) (Marincs and White, 1994) had the *luxCDABE* genes of *Xenorhabdus luminescens* under the control of the *lac* promoter, and was constitutively expressed. All strains used in this study have *E. coli* RFM443 (*strR, galK2, lacΔ74*) as a host. All bacteria except GC2, were grown in Luria-Bertani (LB) medium (Difco Co., USA) supplemented with 25 mg/l of kanamycin monosulfate (Sigma Co., USA) to maintain the plasmid; GC2 was grown using 10 mg/l ampicillin (Sigma Co., USA). The initial pH of the media was adjusted to 7.0 before autoclaving. A single colony of each strain, grown on an LB agar plate with the appropriate antibiotic, was inoculated into 100 ml of sterile LB medium and cultured at 30°C, (37°C for GC2), and 250 rpm in a rotary incubator. When the optical density at 600 nm (OD_{600}) reached 0.8 (late exponential phase), 0.1 ml of the cell broth was transferred into a 96 well test plate and exposed to a different concentration of each pesticide. Finally, bioluminescence (BL), was measured in volumetric light emission (arbitrary units, AU) at set time intervals using a highly sensitive 96 well microplate luminometer (DYNEX Technologies, USA).

All pesticides used in this study, *i.e.*, ziram, ethylene dibromide, benomyl, fenvarelate, methidathion, methoxychlor and glyphosate, were purchased from the Chem-Bio Co. (USA) and are 99% pure. Each pesticide stock solution was prepared at 250 g/l using ethanol as the solvent, except glyphosate, which was prepared in water. In addition, the working solutions for each pesticide were prepared within their range of water solubility. Ethanol was also tested prior to

use to determine its toxic effects on the five *E. coli* strains. Therefore, in this study, the ethanol used for preparing stock solutions was diluted into the media so that its final concentration was less than 0.1 %, which did not result in any significant bioluminescent response (data not shown).

The maximum BL ratio was defined as the ratio of the maximum BL of the induced cells by each pesticide to the maximum BL of the control cells that were exposed to the same concentration of solvent alone. However, in the case of GC2, a constitutive strain was used to determine the relative bioluminescence (RBL), as the ratio of the BL of the induced cells to the BL of the control cells at 120 minutes post induction.

All experiments were performed in triplicate with cells grown separately, for error analysis. The three data points were used to calculate standard deviations, which are represented by error bars.

RESULTS AND DISCUSSION

In this study, it was found that the detection of stresses caused by the seven pesticides might be possible using four different recombinant bacteria capable of detecting specific toxic modes of action. As shown in Fig. 1(a), DPD2794, which is sensitive to DNA damage, showed a dose-dependent response to ziram, a fungicide, but the other strains were unresponsive, indicating that ziram causes DNA damage in the bacterial cells. On the other hand, fenvarelate and glyphosate, shown in Figs. 1(c) and 1(e), respectively, had different effects on the bioluminescence. The insecticide, fenvarelate caused membrane and protein damage, whereas the herbicide, glyphosate caused DNA and oxidative damage in bacteria. In addition, as shown in Table 1, even though ziram, ethylene dibromide and benomyl fell into the same pesticide group, namely fungicides, they were shown to cause different types of stress response in bacteria. Therefore, these results suggest that each pesticide causes different stresses in bacterial cells, and that pesticides may cause specific stresses related to DNA damage, protein damage, oxidative damage, or membrane damage. Moreover, it is apparent that these modes of action are determinable using differences in the response kinetics of these four recombinant bacteria.

It was also found that pesticides cause some cellular toxicity in bacteria. The RBL (relative bioluminescence) is the ratio of the bioluminescence of the cells exposed to each pesticide versus the control. GC2 showed a dose dependent response, via a decreased level of bioluminescence, to ziram, fenvarelate and glyphosate, indicating that these pesticides are toxic to the bacteria (Fig.1(b), (d) and (f)). As well, glyphosate proved to be the most toxic. The effective concentration, which was defined as a 20% (EC_{20}) decrease in bioluminescence after an exposure for 120 minutes, was $4.7\times10^{-4}\mu M$ of glyphosate, while the EC_{50} was $9.1\times10^{-1}\mu M$ (See Table 1). Considering the fact that glyphosate causes oxidative stress at a concentration of $2.7\times10^{-3}\mu M$ of but the EC_{20} is only $4.7\times10^{-4}\mu M$, it would seem that it causes an unknown stress to the bacterial cells.

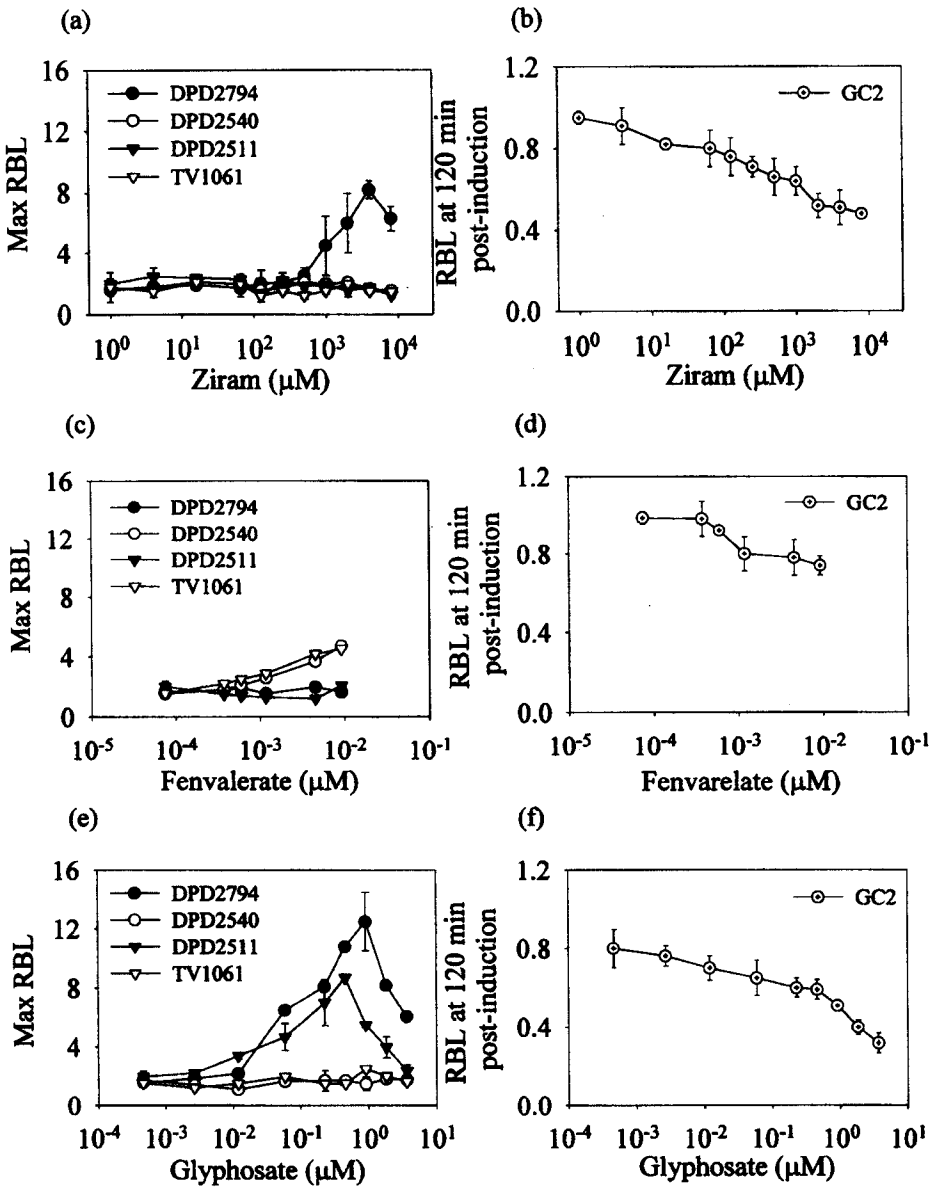

Figure 1. Dose-dependent response curves of five different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for ziram, (c) and (d) for fenvarelate, and (e) and (f) glyphosate. Maximum bioluminescence response means the maximum value of the ratio of the BL in cells exposed to pesticides to the BL of the control cells, and represents the inducibility of BL due to pesticides. In the case of GC2, constitutive strain was used relative bioluminescence (RBL) as the ratio the BL of the induced cells to the BL of the control cells 120 minutes post-induction.

Table 1. General toxicity and stress responses to several pesticides using recombinant bioluminescent bacteria.

		DPD2794 *recA::luxCDABE*		DPD2540 *fabA::luxCDABE*		DPD2511 *katG::luxCDABE*		TV1061 *grpE::luxCDABE*		GC2 *lac::luxCDABE*	
		MDC [μM]	MRC [μM]	MDC [μM]	MRC [μM]	MDC [μM]	MRC [μM]	MDC [μM]	MRC [μM]	EC_{20} [μM]	EC_{50} [μM]
Fungicide	Ziram	1.2×10^{1} (*)	4.0×10^{2} (**)	NR	NR	NR	NR	NR	NR	6.5×10^{1}	2.0×10^{3}
	Ethylene dibromide	NR	NR	0.3×10^{-1} (*)	4.2×10^{2} (**)	NR	NR	NR	NR	2.0×10^{2}	0.3×10^{4}
	Benomyl	NR	NR	NR	NR	NR	NR	3.0×10^{1} (*)	4.7×10^{1} (***)	6.0×10^{2}	6.0×10^{3}
Insecticide	Fenvarelate	NR	NR	1.1×10^{-3} (*)	NT	NR	NR	0.6×10^{-4} (*)	NT	1.1×10^{-3}	NT
	Methidathion	NR	NR	3.3×10^{-2} (*)	2.1×10^{-1} (**)	NR	NR	NR	NR	3.3×10^{-2}	2.1×10^{-1}
	Methoxychlor	NR	NR	0.1×10^{-1} (*)	0.3×10^{-1} (**)	NR	NR	0.6×10^{-2} (*)	NT	0.6×10^{-2}	1.8×10^{-1}
Herbicide	Glyphosate	1.2×10^{-2} (*)	9.1×10^{-1} (***)	NR	NR	2.7×10^{-3} (*)	4.5×10^{-1} (**)	NR	NR	4.7×10^{-4}	9.1×10^{-2}

All concentrations are expressed as μM and the values are significantly different from the control ($p < 0.05$) according to the t-test

MDC : Minimum detectable concentration; Relative bioluminescence = 2.5

MRC : Maximum responsive concentration; The concentration giving the maximum relative bioluminescence (RBLmax), which is defined as the maximum bioluminescence of the sample/Maximum bioluminescence of the control and labeled as *: $2.0 \leq$ RBLmax < 5; **: $5 \leq$ RBLmax < 10; ***: $10 \leq$ RBLmax < 50

EC_{20} : Effective concentration at which the bioluminescence of GC2 decreased by 20% at 120 minutes post-induction

EC_{50} : Effective concentration at which the bioluminescence of GC2 decreased by 50% at 120 minutes post-induction

NR : No response; NT : Not tested

In particular, the herbicide glyphosate was found to be the most toxic with these bacteria, and the insecticides were more toxic than the fungicides, when comparing their EC_{20} and EC_{50} values. Therefore, the level of toxicity of the pesticides followed, in increasing order, the fungicides, insecticides and then the herbicide. In addition, these pesticides appear to cause a relatively mild toxicity to the bacteria, especially when compared with the EC_{20} values seen with other toxicants in our previous studies (Gu et al. 2002; Min et al. 2003).

Typically, it was found that if the EC_{20} value of a chemical was less than nM concentrations, there was about or less than 1 log order of difference between EC_{20} and EC_{50} concentrations. For example, dioxin congeners have EC_{20} and EC_{50} values that are about 5-fold difference, ranged around pM (Min et al. 2003). However, if the EC_{20} value of a chemical was around or above μM concentrations, it typically showed around 2 log orders of difference for a change in the mortality of 30 %, *i.e.*, EC_{20} to EC_{50}. Nonylphenol, naphthalene, and DDT are included in the mildly toxic group because their EC_{20} values and EC_{50} values have large differences between them (Gu et al 2002; Min et al 2003). Therefore, it could be said that the cellular toxicity of such chemicals increase slowly in the bacteria with greater concentrations. Similar results were seen in this study with the pesticides, as shown in Table 1. As well, the results presented in Fig. 1 clearly show and confirm that the mortality decreases slowly with large increases in the pesticide concentrations. In addition, detection of pesticides by four different inducible strains was shown to be very specific, and it was also found to be possible to apply the responses of GC2 to various pesticides to other organisms, since the relative toxicities of the pesticides, the EC_{50} values found in this study, were shown to be somewhat more sensitive compared to the LD_{50} values based on the oral exposure in rats, *i.e.* ziram (0.004 mol/kg), ethylene dibromide (0.0007 mol/kg), benomyl (0.03 mol/kg), fenvarelate (0.08 mol/kg), methidathion (0.018 mol/kg), methoxychlor (0.017 mol/kg) and glyphosate (0.03 mol/kg), especially the insecticides and herbicides. Even though the response mechanisms for the toxicity caused by pesticides is quite different between human and a bacterium, and thus a direct comparison is very difficult, bacteria could be applied as tools in the monitoring and pre-screening of pesticide toxicities for humans because of their sensitivity (Bowmer 1986).

In conclusion, it was found that each pesticide causes different stresses in bacterial cells, and that the toxicity of the pesticides can be measured and compared according to groups of pesticide, *i.e.*, fungicides, insecticides and herbicides. Therefore, the use of the five recombinant bioluminescent bacteria employed in this study provides a quantitative analysis of the different modes of pesticide toxicity.

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