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Evaluation of Toxic Effects Caused by Pesticides in *Escherichia coli* Using Recombinant Bioluminescent Bacteria

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유전자 재조합 발광박테리아를 이용한 농약 독성평가

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요 약

본 연구에서는 유전자 재조합 발광 박테리아를 이용하여 농약에 대한 박테리아의 스트레스 반응과 세포 독성을 분석하였다. 15종류의 농약에 대하여 유전자 손상, 생물막 손상, 산화적 손상 및 단백질 손상을 측정할 수 있는 발광 박테리아와 독성 유무로 인한 세포 독성을 측정할 수 있는 발광 박테리아, 5종을 이용하여 스트레스 반응을 분류하고 세포 독성 정도를 분석하였다. 그 결과, 농약의 화학적 구조가 박테리아의 스트레스 반응에 영향을 미치며, 산화과정이 진행 됨에 따라 독성의 작용 기작이 변하는 것을 확인할 수 있었다.

이와 같은, 유전자 재조합 발광 박테리아를 이용한 생물체내의 독성 메커니즘에 대한 분석은 생태계 유해물질들에 의한 독성을 분석하고 예상하기 위해 적용될 수 있을 것이다.

Key words : *E. coli*, pesticides, recombinant bioluminescent bacteria, structure, oxidation

INTRODUCTION

Environmental pollution problems caused by pesticides have steadily increased. Numerous methodologies, therefore, have been developed for sensitive and effective environmental monitoring. However, analytical techniques require complex and expensive methods employing GC/MS and LC/MS. Moreover, toxicity data for the tested sample cannot be obtained from the analytical techniques. The most commonly used toxicity bioassays were developed by using higher organisms such as daphnia or fish. The major drawback of the bioassay is the long detection time, in the range of days to weeks. Thus, the development of the toxicity biosensor using microorganisms, including bioluminescent bacteria, has drawn heavy attention from researchers since it offers high sensitivity, low cost,

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and a rapid response. The commercially available bioassay, Microtox^{TM}, was developed on the basis of luminous bacteria. Toxicity to bacterial cells is measured through a lowered bioluminescent emission. With the development of recombinant DNA technology, many different recombinant bioluminescent bacteria have been constructed with one of the methods adopted being the use of a fusion between the bacterial *lux* operon and stress promoters. These 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 very wide range of promoters are known to exist in the prokaryotic cells and fusion of stress promoters with the *lux* genes results in new cellular biosensing strains which emit light when stressed. In addition, using bioluminescence as a reporter has several advantages, such as in vivo, real-time, on-line measurement.

MATERIALS AND METHODS

1. Strains, growth, culture media and bioluminescence measurement

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. EBHJ1 (*sodA::luxCDABE*) (Lee and Gu, 2001), EBJM1 (*pqi-5::luxCDABE*) (Ahn and Gu, 2002) were constructed at national research laboratory on environmental biotechnology in Gwangju Institute of Science and Technology (GIST) and also used in this study.

All bacteria except GC2 were grown in a Luria-Bertani (LB) medium (Difco Co., USA) supplemented with 25 mg/L kanamycin monosulfate (Sigma Co., USA) to maintain the plasmid, GC2 was grown with 10 mg/L ampicilin (Sigma Co., USA). The pH of the media was adjusted to 7.0. A single colony of each strain, grown on an LB agar plate with the appropriate antibiotics, was inoculated into 100 mL of sterile LB medium and cultured at 30℃, (37℃ for GC2), and 250 rpm in a rotary incubator (Perkin-Elmer Co., USA). Sterilized each flask was inoculated with 2 mL of inoculum from a seed flask previously cultivated for 8 hours in 100 mL of medium. When the optical density at 600 nm (OD_{600}) reached to 0.08 (the early-exponential growth phase), pesticides were added to the culture media. Emitted bioluminescence (arbitrary units, AU) was monitored using a highly sensitive 96well microplate luminometer (DYNEX Technologies, USA) and cell growth was measured with a UV/Vis spectrophotometer (Perkin-Elmer Co., USA) at 600 nm.

2. Chemicals

In this study, 15 pesticides were tested. All of the pesticides used in this study, aldrin, dieldrin, isodrin, endrin, endosulfan, endosulfan sulfate, carbofuran, carbofuran-7-phenol, parathion, 3-methyl-4-nitrophenol, 2,4-D, chloroacetaldehyde, acetaldehyde, sodium arsenite and sodium arsenate were purchased from Sigma Co. (USA). Because most pesticides are not soluble in water, ethanol was used as a solvent and diluted into media so that the final concentration of ethanol was less than 2%, which did not result any significant bioluminescence responses in the 96well luminometer. Sodium arsenite and sodium arsenate are soluble in water. The test concentrations for each pesticide were decided based on the water solubility of each chemical.

3. Data analysis

Most pesticides are not soluble in water. Therefore, ethanol was used as a solvent and diluted into media so that the final concentration of ethanol was less than 2%, which did not result in any significant bioluminescence responses in the 96well luminometer.

In inducible strains BL (bioluminescence) increases dose-dependently until the cells can adjust them-

Table 1. Stress responses and cellular toxicities to several pesticides using recombinant bioluminescent bacteria

	DPD2794 *recA::luxCDABE*		DPD2540 *fabA::luxCDABE*		DPD2511 *katG::luxCDABE*		TV1061 *grpE::luxCDABE*		GC2 *lac::luxCDABE*
Pesticides: Conc.[ppm]	MDC[a]	MRC[b]	MDC	MRC	MDC	MRC	MDC	MRC	EC_{20}[c]
Aldrin	10(*)	400(*)	200(*)	400(*)	-	-	-	-	-
Dieldrin	-	-	10(***)	10(***)	100(*)	100(*)	100(***)	100(***)	-
Isodrin	-	-	100(**)	100(**)	-	-	10(***)	10(***)	-
Endrin	-	-	10(***)	10(***)	-	-	-	-	-
Parathion	-	-	10(***)	100(***)	-	-	-	-	-
2,4-D	-	-	-	-	-	-	-	-	400
3-Methyl-4-nitrophenol	-	-	-	-	10(**)	10(**)	-	-	0.01
Acetaldehyde	-	-	-	-	-	-	-	-	-
Chloroacetaldehyde	-	-	10(*)	10(*)	-	-	-	-	$1<EC_{20}<10$
Arsenite	-	-	-	-	-	-	-	-	$1<EC_{20}<10$
Arsenate	-	-	-	-	-	-	-	-	40
Endosulfan	-	-	-	-	-	-	-	-	-
Endosulfan sulfate	-	-	-	-	-	-	-	-	-
Carbofuran	-	-	-	-	-	-	-	-	-
Carbofuran-7-phenol	-	-	-	-	-	-	-	-	100

a. MDC : Minimum detectable concentration; Relative bioluminescence = 2.5
b. MRC : Maximum responsive concentration; The maximum response for stress inducible bioluminescent bacteria (RBLmax): Maximum bioluminescence of the sample/Maximum bioluminescence of the control (* : 2.0≤RBLmax 5; ** : 5≤RBLmax<10; *** : 10<RBLmax≤50)
c. EC_{20} : Effective concentration at which the bioluminescence decreased by 20% at 120 minutes post-induction
e. – : No response

selves to a new environment when they are exposed to toxicity caused by pesticides. BL decreases after it marks a climax.

The RBL (relative bioluminescence) was used to evaluate the stresses and toxicities caused by pesticides. The RBL is defined as ratio of the BL of the induced cells by each pesticide to the BL of control cells that were exposed to the same concentration of the solvent alone. The maximum RBL is the RBL when the bacteria show the maximum bioluminescence.

In the case of a constitutive strain, GC2, EC_{20} value (the concentration at which the bioluminescence decreased by 20%) was used. Usually the RBL values are obtained from BL within 1 hr. Especially RBL values were calculated from the BL at 200 min after post-induction in the test of sodium arsenite and sodium arsenate because of their delayed respon-ses.

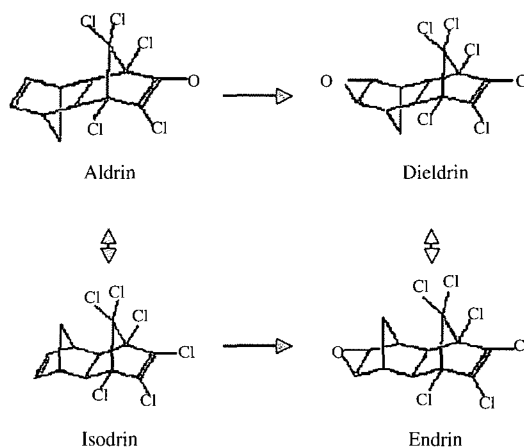

Fig. 1. A isomeric form of aldrin is isodrin and endrin is also a isomeric form of dieldrin. Aldrin is slowly transformed to dieldrin and isodrin also undergos microbial oxidation to endrin by a mechanism analogous to the biooxidation of aldrin to dieldrin.

RESULT AND DISCUSSION

The responses of the strains to each of the chemicals are compiled in Table 1. It was found that the detection of stresses caused by the pesticides might be possible using four different recombinant bacteria

capable of detecting specific toxic modes of action. In addition, it was also found that pesticides cause some cellular toxicity in bacteria.

In the test of drin pesticides, it was found that chemical isomers cause different stresses in bacteria and the oxidation processes change the toxic mode of action by pesticides. Aldrin, dieldrin, isodrin and endrin are included in cyclodiene series of organochlroinated insecticides. The use or production of these pesticides is prohibited. However, the residual pesticides and the use in some underdeveloped countries can be problems. Previous uses of drin pesticides and their persistence have toxic effects on ecosystem health. Therefore, in this study drin pesticides were tested with recombinant bioluminescent bacteria. One commonality of all the drin pesticides was that they caused membrane damage. However, four recombinant bioluminescent bacteria showed different stress responses for each drin pesticide depending upon their chemical structures and oxidation. Aldrin and isodrin are isomers (Fig. 1). Even though they have same molecular formula and same molecular weight, the difference of their structures caused different stresses to the bacterial cells. Aldrin mainly caused membrane damage but isodrin mainly caused protein damage. In addition, aldrin was also related to the DNA damage but isodrin was not. In the test of dieldrin and endrin, dieldrin caused

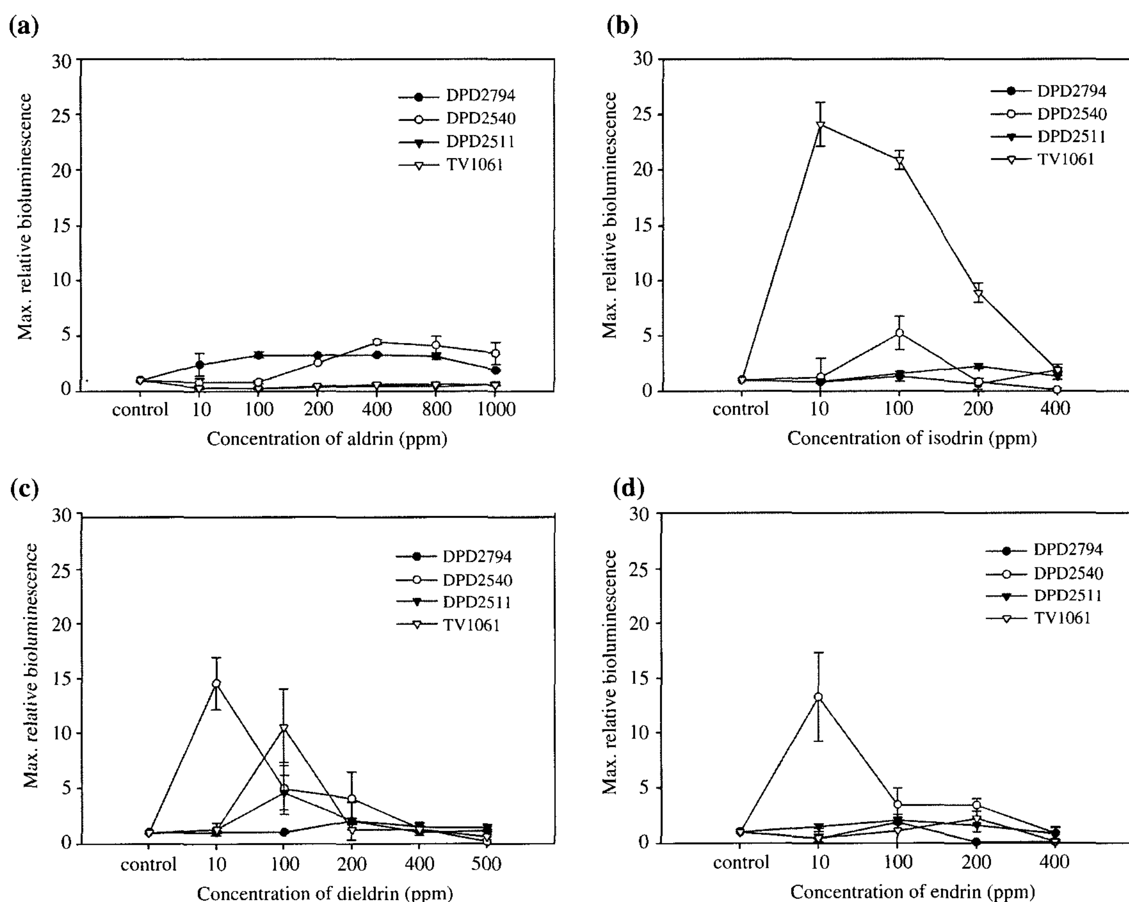

Fig. 2. Dose-dependent response curves of four different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061) for drin pesticides, (a) aldrin, (b) isodrin, (c) dieldrin and (d) endrin. Maximum relative bioluminescence means the maximum value of the ratio of the BL of cells exposed to pesticides to the BL of the control cells, and represents the inducibility of BL due to pesticides.

membrane, protein and oxidative damages but endrin just caused membrane damage.

Meanwhile, the stresses caused to the bacteria were changed by the oxidation of chemicals. DNA damage was not detected with dieldrin, the oxidated form of aldrin, but dieldrin caused protein and oxidative damages to the bacteria. Isodrin was highly related to the protein damage but the oxidated form, endrin, did not cause protein damage (Fig. 2). However, the constitutive strain, GC2, did not show dose-dependent decreases of RBL value with four drin pesticides (Fig. 3).

The use of chloroacetaldehyde covers a wide range of industry and is used as a fungicide in agriculture. Fig. 4 (a) showed that chloroacetaldehyde only caused DNA damage in the bacterial cells. This result corresponds with the fact that chloroacetaldehyde is a kind of mutagens (J. Steven *et al.*, 1988; Zdenka *et al.*, 1992). The other inducible strains did not show significant responses with chloroacetaldehyde. On the other hand, acetaldehyde, shown in Fig. 4 (c), did not cause DNA damage in bacteria. This means that the -Cl of chloroacetaldehyde plays an important role in causing DNA damage in bacteria. For acetaldehyde, all the four recombinant bioluminescent bacteria did not show significant responses in bacteria. The constitutive strain, GC2, showed dose-dependent decrease of bioluminescence just to chloroacetaldehyde not acetaldehyde (Fig. 4 (b) and (d)). This result also

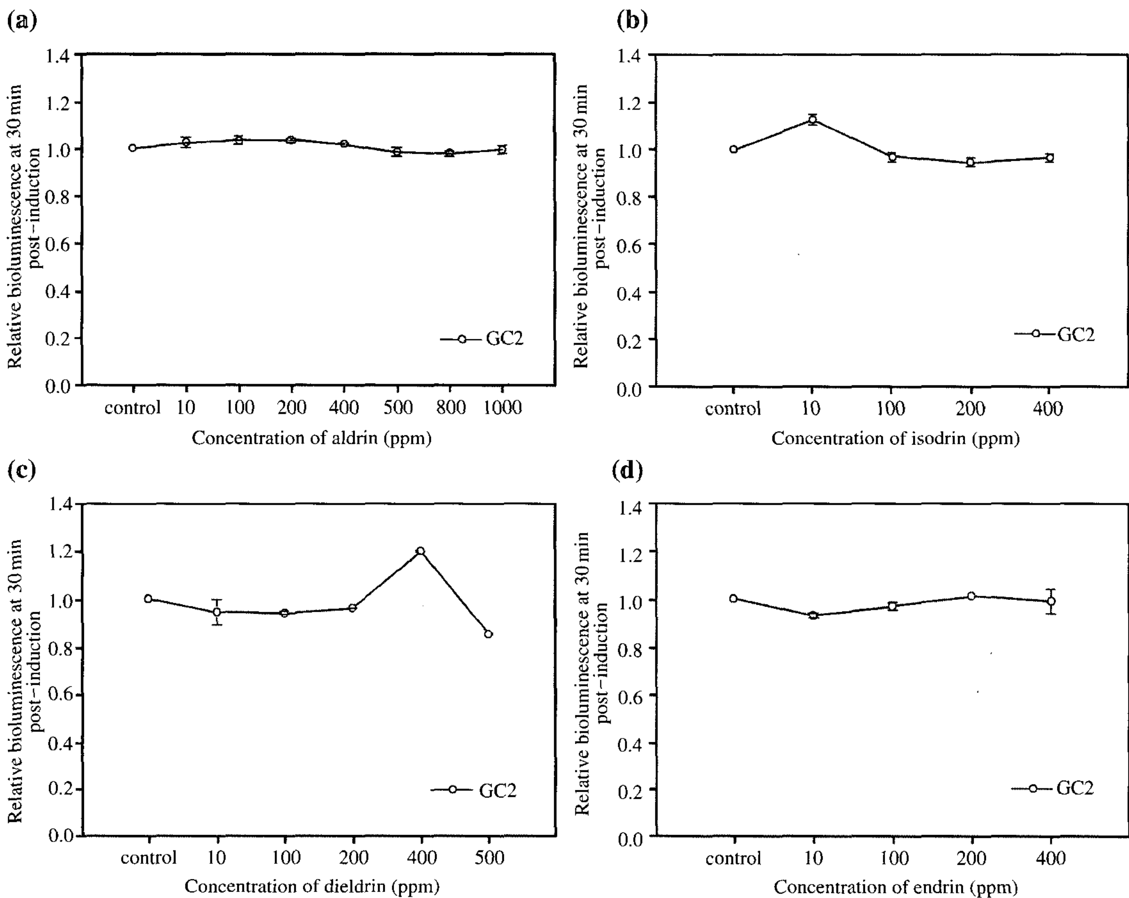

Fig. 3. Response curves of GC2, the constitutive strain, for drin pesticides, (a) aldrin, (b) isodrin, (c) dieldrin and (d) endrin. Relative bioluminescence (RBL) was used after an exposure for 30 minutes.

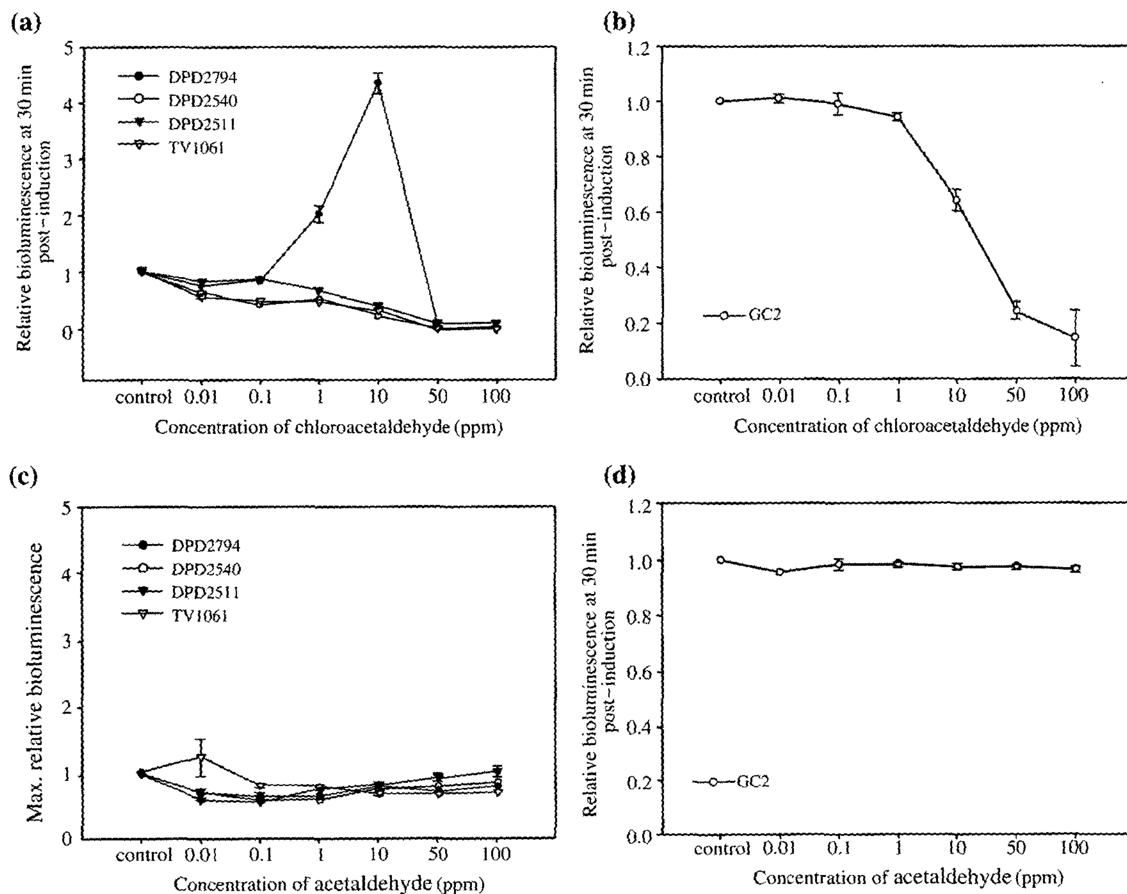

Fig. 4. Response curves of five different recom-binant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for chloroacetaldehyde, (c) and (d) for acetaldehyde.

suggests that the -Cl of chloroacetaldehyde cause toxicity in bacteria.

As shown in Fig. 5. (a), DPD2540, which is sensitive to membrane damage, showed a dose-dependent response to parathion, a kind of organophosphate, but the other strains were unresponsive, indicating that parathion causes membrane damage in the bacterial cells. On the other hand, 3-methyl-4-nitrophenol, a major decomposition product of fenitrothion which is also a kind of organophosphate pesticides, had different effect on the bioluminescence. The strain, DPD2511 (*kat::luxCDABE*) which is sensitive to H_2O_2, using *katG* promoter under the control of OxyR, showed a dose-dependent response to the 3-methyl-4-nitrophenol (Fig. 5). The response was compared with those of EBHJ1 and EBJM1 which have promoters responsive to induction by a transcriptional activator SoxRS. The EBHJ1 and EBJM1 didn't give any significant response to 3-methyl-4-nitrophenol (Fig. 6). This means that 3-methyl-4-nitrophenol activates the OxyR protein and the oxidized form of OxyR binds to the *katG* promoter where it stimulates transcription. From the results of parathion and 3- methyl-4-nitrophenol tests it was found that each pesticide involved in the same group based upon the composition of pesticides caused different stresses in the bacteria.

The four recombinant bioluminescent bacteria

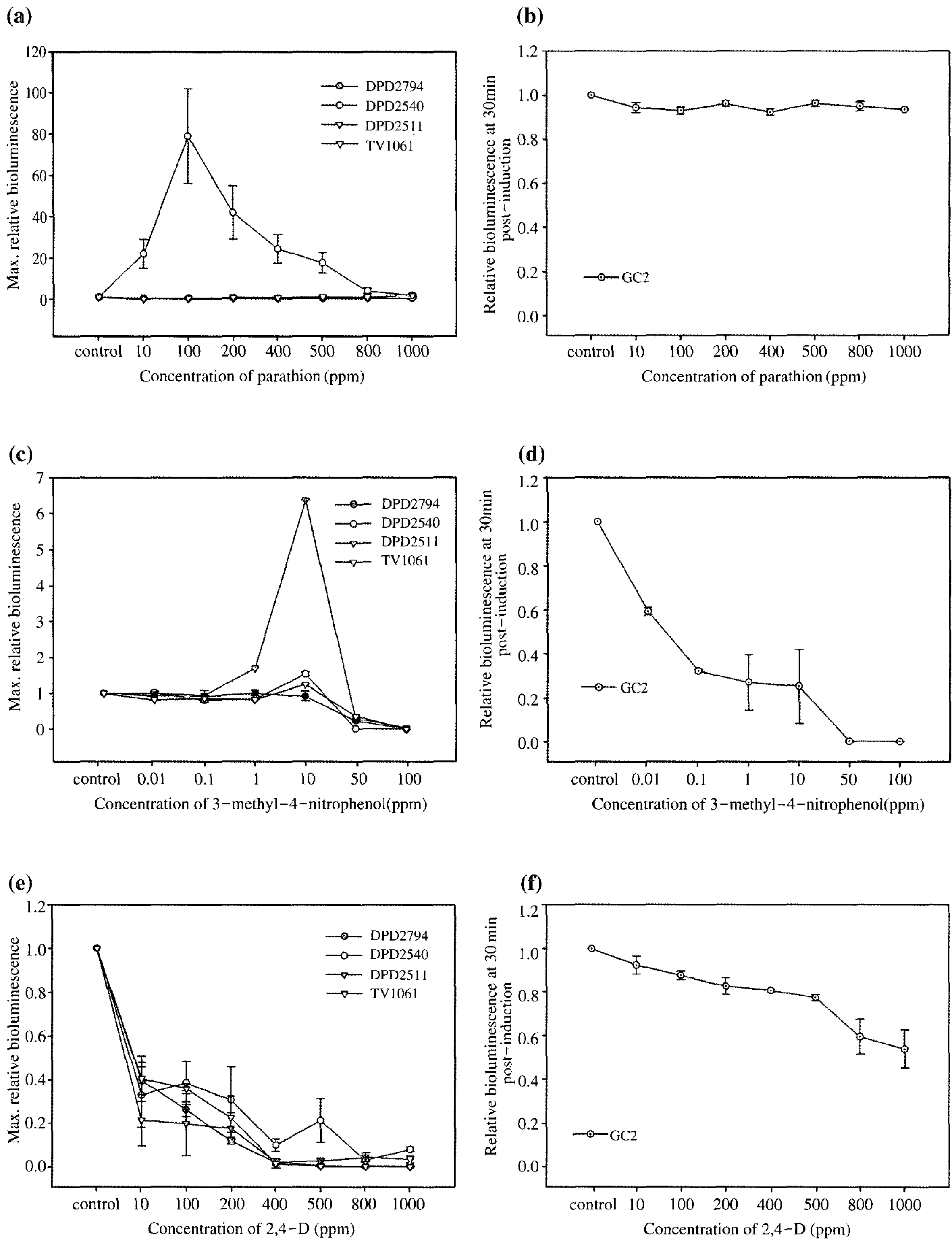

Fig. 5. Response curves of five different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for parathion, (c) and (d) for 3-methyl-4-nitrophenol, and for (e) and (f) for 2,4-D.

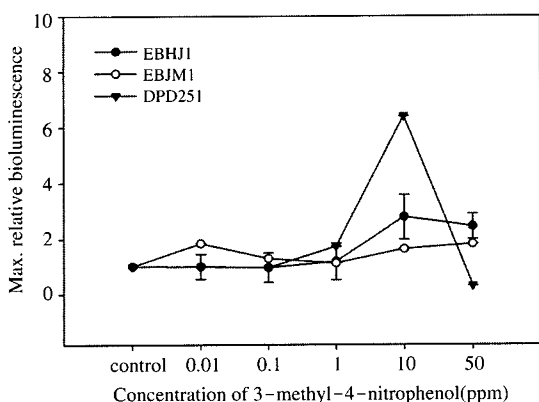

Fig. 6. Response curves of three diffrent strains detecting oxidative stresses for 3-methyl-4-nitrophenol.

showed no significant responses with a well known herbicide, 2,4-D. It was also found that pesticides cause some cellular toxicity in bacteria. GC2 showed dose dependent responses, via decreased levels of bioluminescence, to 3-methyl-4-nitrophenol and 2,4-D, indicating that these pesticides are toxic to the bacteria (Fig. 5).

Considering the fact that 3-methyl-4-nitrophenol caused oxidative stress at a concentration of 10 ppm but the EC_{20} was less than 0.01 ppm, it would seem that it caused an unknown stress to the bacterial cells.

In this study, toxicities of arsenite and arsenate were tested with water soluble arsenic compounds, sodium arsenite and sodium arsenate. As shown in

Fig. 7. Response curves of five different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for arsenite, (c) and (d) for arsenate.

Fig. 7. (a) and (c), both arsenite and arsenate did not cause significant stresses in the bacteria. However, they caused some cellular toxicity in the bacteria. The EC_{20} was between 1 and 10 ppm for arsenite and 40 ppm for arsenate (See Table 1). This result was consistent with the fact that arsenite is more toxic than arsenate.

Endosulfan and its derivative, endosulfan sulfate did not cause any significant stresses in bacteria. In GC2, they did not cause cellular toxicity. In the test of carbofuran and its decomposition product, carbofuran-7-phenol, four inducible strains did not show any significant responses. In the case of carbofuran-7-phenol, EC_{20} value was about 100 ppm, suggesting that this byproduct of carbofuran degradation may be more toxic in bacteria.

CONCLUSION

In this study, the toxicities of various pesticides were tested with recombinant bioluminescent bacteria. The responses of the strains to each of the chemicals are compiled in Table 1. It was found that the detection of stresses caused by the pesticides might be possible using four different recombinant bacteria capable of detecting specific toxic modes of action. In addition, it was also found that pesticides cause some cellular toxicity in bacteria.

In the test of drin pesticides, it was found that

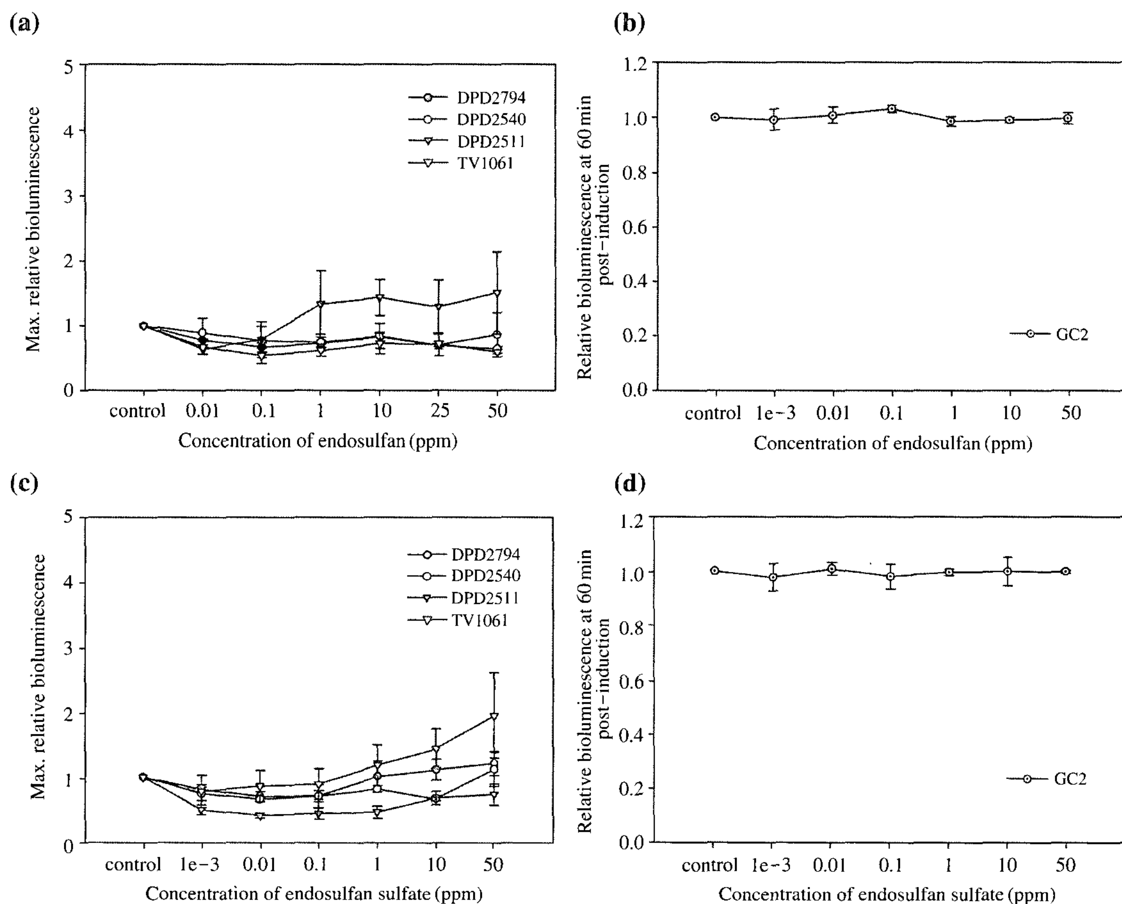

Fig. 8. Response curves of five different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for endosulfan, (c) and (d) for endosulfan sulfate.

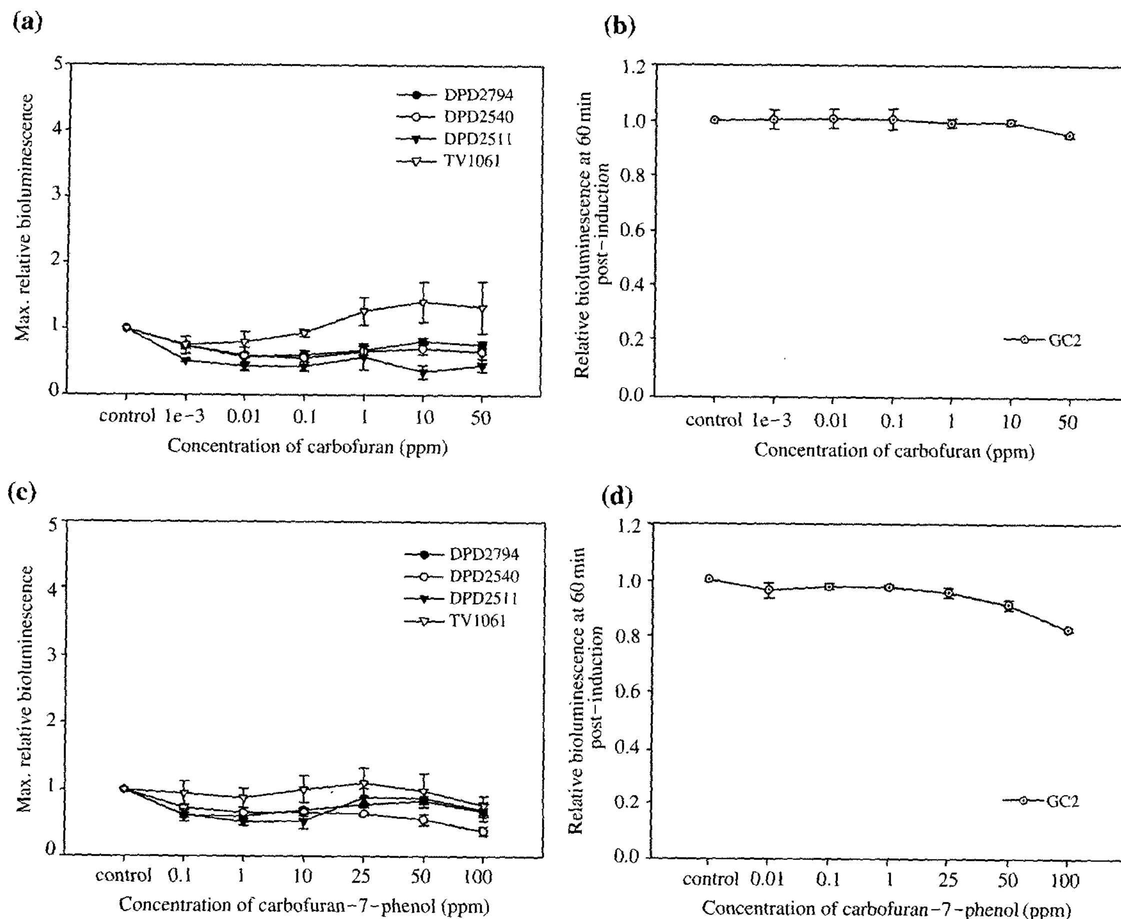

Fig. 9. Response curves of five different recombinant bioluminescent bacteria (DPD2794, DPD2540, DPD2511, TV1061 and GC2) for (a) and (b) for carbofuran, (c) and (d) for carbofuran-7-phenol.

chemical isomers cause different stresses in bacteria and the oxidation processes change the toxic mode of action by pesticides. The test of parathion and 3-methyl-4-nitrophenol suggested that stresses and cellular toxicities caused by pesticides in the same group might be changed. Meanwhile, in the test of chloroacetaldehyde and acetaldehyde, it was found that the functional group -Cl plays important role in causing DNA damage and cellular toxicity.

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