Effect of 2,4,5-Trichlorobiphenyl (PCB-29) on Oxidative Stress and Activities of Antioxidant Enzymes in Tomato Seedlings

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ABSTRACT: Leaves of two-week old seedlings of tomato (*Lycopersicon esculentum*) were treated with various concentrations (0, 0.2 and 0.4 μg/l) of 2,4,5-trichlorobiphenyl (PCB-29) and subsequent growth of seedlings, symptoms of oxidative stress and activities of antioxidant enzymes were investigated. Compared with the non-treated control, foliar application of PCB-29 decreased both biomass and superoxide (O_2^-) radical production but increased hydrogen peroxide production and lipid peroxidation such as malondialdehyde (MDA) formation with increased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). Further studies on the isozymes of SOD, peroxidase (POD) and APX showed that all three isozymes of SOD such as Mn-SOD, Fe-SOD and Cu/Zn-SOD, two among four isozymes of POD and all three isozymes of APX were selectively increased in response to PCB. Therefore, we suggest that a possible cause for the reduction of seedling growth by PCB exposure is the oxidative stress including over production of hydrogen peroxide and the selective expression of specific isozymes of some antioxidant enzymes.

Key words: Antioxidant enzymes, Hydrogen peroxide, Oxidative stress, PCB

INTRODUCTION

Polychlorinated biphenyls (PCBs) are industrial compounds and are ubiquitous environmental contaminants (Bedard *et al.* 1980), which produce a wide range of toxicities with various degree of chlorination (Pearson *et al.* 1996, Jordan and Feeley 1999). Several mechanisms have been proposed for the toxicity of PCBs and related chemicals particularly in animals, with oxidative stress including free radical production, lipid peroxidation and DNA damage is being considered as one of the important ones (Stohs *et al.* 1991, Hassoun *et al.* 2000). In plants, a few studies have been demonstrated that PCBs are accumulated (Jensen *et al.* 1992, Bobovnikova *et al.* 2000, Krauthacker *et al.* 2001). However, the effect of PCB on plant growth and its mechanism(s) of action are not well known.

One of the biochemical changes occurring in plants subjected to various environmental stress conditions is the production of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide, singlet oxygen and hydroxyl radicals ($\cdot OH$) (Hernandez *et al.* 1993, Mishra *et al.* 1995, Iturbe-Ormaetxe *et al.* 1998, Cho and Park 2000). The ROS have a role in lipid peroxidation, membrane damage and consequently in plant senescence (Fridovich 1986, Thompson *et al.* 1987, Breusegem *et al.* 2001), and antioxidant enzymes such as superoxide dismutase (SOD), peroxidases (POD) and catalases (CAT) are involved in

the scavenging of ROS (Asada 1992, Foyer 1993). SOD is a metalloprotein that catalyzes the dismutation of superoxide to H_2O_2 and molecular oxygen (Salin 1987, Allen 1995). SOD enzymes are classified according to their metal cofactor and their subcellular localization. The predominant forms are a mitochondrial Mn-SOD, a cytosolic Cu/Zn-SOD, a chloroplastic Cu/Zn-SOD, and a chloroplastic Fe-SOD. Various antioxidant enzymes such as CAT and POD eliminate H_2O_2. CAT found predominantly in peroxisomes dismutase H_2O_2 into H_2O and O_2, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Sudhakar *et al.* 2001). Ascorbate peroxidase (APX) is primarily located in both chloroplasts and cytosol and as the key enzyme of the ascorbate cycle, and eliminates peroxides by converting ascorbic acid to dehydroascorbate (Asada 1992). As a member of the ascorbic acid-glutathione cycle, APX is one of the most important enzymes playing a crucial role in eliminating toxic H_2O_2 from plant cells (Foyer *et al.* 1994).

The objective of present study is to investigate whether PCB induce phytotoxicity expressed as growth inhibition and the phytotoxicity is mediated by oxidative stress in tomato seedlings. Physiological and biochemical analyses including activities and isozyme analysis of various antioxidant enzymes will provide new insights into the processes of PCB toxicity in plants. The data show that tomato seedlings, which have been used as a

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research model to understand oxidative stress (Mazhoudi *et al.* 1997, Cho and Park 2000), produce H_2O_2 and the activities of related antioxidant enzymes are altered in response to PCB exposure. This information is pre-required to understand the uptake, persistence and distribution of PCBs in plants. Further, the resistant plants capable of storing, degrading or detoxifying PCBs and the susceptible plants might be used in the phytoremediation (Estime and Rier 2001) and indicators (Eriksson *et al.* 1989) of a PCB-contaminated environment, respectively.

MATERIALS AND METHODS

Plant culture and treatments

Seeds of tomato (*Lycopersicon esculentum* Mill) were germinated and incubated on MS medium solidified with 0.3% Phytagel (Sigma, USA) at 25°C with 12 h of light (250 μM m⁻²s⁻¹). For PCB treatment, 50 μl of 0, 0.2 or 0.4 μg/l of PCB-29 (2,4,5-trichlorobiphenyl, Cambridge Isotope Laboratories, USA) were applied directly on the leaves of two-week old seedlings and incubated for up to 10 days. Before application, PCB in isooctane was mixed with 0.05% Tween-20. After blowing the isooctane to dryness, the Tween-20 containing PCB was dissolved in water and brought to final volume (Isensee and Jones 1971). For biomass measurement, seedlings were collected on day-10 after PCB treatment, dried for 48 h at 80°C and weighed. For ROS production, lipid peroxidation and enzyme activity assays, leaves from each treatment were pooled and analyzed on day-5 after foliar PCB application.

Analysis of lipid peroxidation

The level of lipid peroxides in the samples was determined as malondialdehyde (MDA) content. MDA was determined by thiobarbituric acid reaction as described by Dhindsa *et al.* (1987), and the concentration of MDA is calculated based on A_{532}-A_{600} (ε = 155 mmol/cm).

Determination of O_2^- and H_2O_2 levels

Fresh leaves (200mg) were pooled and homogenized with a pestle in an ice-cold mortar in 2 ml of cold 0.2M sodium phosphate buffer (pH 7.2) containing 1mM diethyldithiocarbamate to inhibit SOD activity. The homogenate was centrifuged at 4,000xg for 10 min, and the supernatant was collected. O_2^- content was measured by following the increase in absorbance at 540nm caused by NBT reduction (Chaitanya and Naithani 1994). Reaction mixture included 1ml 0.25mM NBT and 100μl supernatant.

The degree of inhibition of aconitase activity in the presence of H_2O_2 was used as a specific marker of levels of H_2O_2 (Zhang and Kirkham 1996). Fresh leaves (200mg) were ground with a pestle in a mortar in 2 ml of an ice-cold grinding buffer containing

3mM cysteine, 5mM EDTA, 1 g/l bovine serum albumin, and 10mM MOPS [3-(N-morpholino)-propanesulfonic acid], adjusted to pH 7.4 with NaOH. The homogenate was filtered through 0.45 μm filter (Millipore, USA), and the filtrate was centrifuged at 10,000 x g at 4°C for 10 min. The supernatant was collected and used for the assay of aconitase activity. The reaction mixture contained 100μl 0.148 M cis-aconitate (pH 6.5), 620μl grinding buffer, 100μl 2.5 M NADP, 20μl (0.4 units) NADP-dependent isocitrate dehydrogenase, and 200μl supernatant. Aconitase activity was calculated by following the increase in absorbance at 340nm caused by NADPH formation and using the NADPH extinction coefficient of 6.2 mM/cm.

Assays of SOD, APX, GPX and CAT activities

All leaf samples except for APX activity were ground with liquid nitrogen and homogenized with extraction buffer containing 10 mM potassium phosphate buffer (pH 7.8), 0.5% triton X-100 and 1.0% polyvynylpyrrolidone. For APX, leaves were homogenized and extracted according to the method of Lee and Lee (2000). The extracts were centrifuged at 12,000 x g for 15 min, and protein concentration of the supernatant was determined using the Bradford dye-binding assay (Bradford 1976) with bovine serum albumin as a standard protein. For SOD measurement, assay mixture included 50mM potassium phosphate buffer (pH 7.8), 0.1mM EDTA, 50 μM xanthine, 10μM ferricytochrome C, xanthine oxidase (enough to cause between 0.03 and 0.04 cause at A_{550}/min), and 100μl of protein extract. One unit of enzyme activity is the amount that inhibits the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 1ml reaction volume (McCord and Fridovich 1969). The activity of APX was assayed by following the decrease in absorbance at 290nm caused by ascorbate oxidation (Nakano and Asada 1981). CAT activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240nm (Beers and Sizer 1952). One unit of CAT is defined as the amount necessary to decompose 1 μmol of H_2O_2 per minute at 25°C. GPX activity was assayed according to the modified method of Shah *et al.* (2001) using extinct coefficient of 26.6 mM/cm for 1 min. Enzyme specific activity is defined as (mol of H_2O_2 reduced per minute (mg/protein).

Analyses of SOD, POD and APX isozymes

For SOD, the protein samples (50μg) were separated by native PAGE on a separating gel of 12% (w/v) polyacrylamide in a tank buffer containing 25mM Tris (pH 8.3) and 192mM glycine. After incubation for 30 min in 50 mM potassium phosphate buffer (pH 7.0) containing 3mM KCN (inhibitor of the Cu/Zn-SOD) or 2mM H_2O_2 (inhibitor of the Fe-SOD and Cu/Zn-SOD) (Lee and Lee 2000), the gels were stained for 30 min in the dark using a 1:1 mixture of (a) 0.06mM riboflavin and 0.651% (w/v) TEMED,

and (b) 2.5mM nitroblue tetrazolium (NBT), both in 50mM phosphate buffer at pH 7.8 and developed for 20 min under light conditions (Mckersie et al. 2000). After staining, the gels were photographed with a digital camera (Nikon Coolpix-990, Japan), and an analysis program (Labworks image acquisition and analysis software Ver. 4.0.0.8, Media Cybernetics, USA) was used to measure the intensity of each band. The area of individual SOD isozymes was expressed relative to a standard of Escherichia coli Fe-SOD (Sigma Chemical, St. Louis) to calculate each enzyme activity. For POD, the protein samples (50μg) were separated by native PAGE on a separating gels of 10% (w/v) polyacrylamide in a tank buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine, and the band formation due to the formation of oxidized amino-ethyl-carbazole during H_2O_2 breakdown by peroxidase was detected and photographed immediately (Manchenko 1994). Isozymes of APX were identified with the method described by Lee and Lee (2000) except the use of 12.5 mM NBT for 60 min instead of 2.45 mM for 10-20 min at the last procedure. The activity gels were photographed and the band intensities of isozymes were measured by gel analysis software (SigmaGel Ver. 1.0. SPSS, USA).

Statistics

The data are the means ± SE of three independent replicates. The analyses of variance were computed on statistically significant differences determined based on the appropriate F-tests. The mean differences were compared utilizing Duncan's multiple range test.

RESULTS AND DISCUSSION

Growth response, ROS production and lipid peroxidation

The effect of PCB-29 on seedling growth expressed as dry weight, O_2^- and H_2O_2 production, and lipid peroxidation is shown in Table 1. Compared with the non-treated control, PCB exposure with both 0.2 and 0.4 μg/l induced a significant reduction of dry weight measured on day-10 after PCB treatment. To know whether oxidative stress including lipid peroxidation and ROS production were involved in the reduction of seedling growth, production of MDA as the decomposition product of polyunsatu-

rated fatty acids, O_2^- radical and relative levels of H_2O_2 estimated from the degree of inhibition of aconitase activity were investigated. Compared with the control, PCB exposure significantly increased MDA and H_2O_2 but decreased O_2^- levels in leaves. Compared with the control, the significantly decreased activities of aconitase in leaves implied that H_2O_2 levels were increased with PCB exposure.

Although a few studies have been demonstrated that PCBs are accumulated in plants (Jensen et al. 1992, Bobovnikova et al. 2000, Krauthacker et al. 2001), the absorption pathways and the pattern of plant growth after PCB exposure have not been described yet. Although the accumulation of PCB in plant tissues and the mechanism of penetration of exogenous PCB into tissue are not known at present, the waxy surface of leaves has the ability to absorb lipophilic pollutants (Krauthacker et al. 2001). Due to the hydrophobic nature of PCBs, they will adsorb onto cellular lipids whether the plants are alive or dead (Fletcher et al. 1987). The observed changes in the biomass of tomato seedlings might be due to both the reduction of chlorophyll contents in leaves (data not shown) and tissue or cell damage as indicated by lipid peroxidation. A consistent increase in MDA level measured on day-5 paralleled an increase of exposure level of PCB. Destruction of lipid components of membrane by lipid peroxidation causes membrane impairment and leakage (Thompson et al. 1987).

Activities of SOD, CAT, GPX and APX

Foliar PCB application altered the activities of antioxidant enzymes including SOD, CAT, GPX and APX (Table 2). Compared with the control, the total activities of SOD, GPX and APX measured on day-5 significantly increased with PCB exposure. However, the CAT activity maintained stable with 0.2 μg/l and decreased significantly with 0.4 μg/l PCB.

Free radical reactions have been suggested to play an important role in the degradation process of membrane polar lipids in senescence, and increased level of MDA after PCB exposure might indicate the presence of ROS (Thompson et al. 1987). ROS are effective at different levels of stress-induced deterioration (Foyer et al. 1994). Since SOD located in various compartments is a major scavenger of O_2^- radicals and catalyzes the disproportionate of two O_2^- radicals to H_2O_2 and O_2 (Salin 1987), the

Table 1. Dry weights of seedlings collected on day-10, superoxide radical and hydrogen peroxide (aconitase activity) levels and lipid peroxidation in leaves of tomato seedlings collected on day-5 after PCB treatment. Values are means ± SE of at least three independent replicates. Values in a column followed by the same letter are not significantly different at the 0.05 levels according to Duncan's multiple range tests

PCB (μg/l)	Dry weight (mg/plant)	O_2^- ($\triangle A_{540}$/min/g FW)	Aconitase (nmol/min/g FW)	MDA (nmol/g FW)
0	1.46±0.10a	0.61±0.02a	17.46±1.00a	7.20±0.16a
0.2	1.17±0.10b	0.44±0.04b	13.05±3.00b	9.60±0.16b
0.4	1.13±0.20b	0.45±0.01b	11.82±0.90c	13.5±1.00c

Table 2. Antioxidant enzyme activities of leaf extracts from tomato seedlings collected on day-5 after PCB treatment. Values are means ± SE of at least three independent replicates. Values in a column followed by the same letter are not significantly different at the 0.05 levels according to Duncan's multiple range tests

| PCB (μg/l)	SOD	CAT (units/mg protein)	GPX	APX
0	296.10± 7.80a	6.65±0.20a	1.07±0.14a	9.30±1.00a
0.2	364.05± 2.05b	6.06±0.10a	0.97±0.07a	16.20±3.50b
0.4	350.00±11.30b	5.73±0.10b	1.36±0.05b	21.80±3.60c

observed increase in SOD activity after PCB exposure could increase the ability of the seedlings to scavenge O_2^- radicals that is highly reactive, forming hydroperoxides with enes and dienes (Salin 1987, Halliwell and Gutteridge 1989). In fact, after PCB-exposure, O_2^- content in seedlings was much lower and H_2O_2 content was much higher than in control (Table 1). The decreased O_2^- and the increased H_2O_2 contents with PCB-exposure might be induced by the increased dismutation activity of SOD: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$.

The activity of APX was proportionally increased with higher PCB level (Table 2). Since APX is primarily located in chloroplasts and cytosol and functions as the key enzyme of the glutathione-ascorbate cycle eliminating peroxides by converting ascorbic acid to dehydroascorbate (Asada 1992, Foyer et al. 1994), the increased APX activity might be due to the increased H_2O_2 production (Table 1) resulting from the increased activity of SOD. Total activity of GPX using guaiacol as a substrate was increased with 0.4 μg/l PCB but not with 0.2 μg/l PCB, indicating less sensitivity of GPX to PCB than APX. Meanwhile, the levels of catalase activity decreased with PCB exposure (Table 2). The decline might be due to the activity found predominantly in peroxisomes (Fadzilla et al. 1996, Sudhakar et al. 2001) and less accessibility to PCB-induced H_2O_2.

Isozymes of SOD, POD and APX

Using KCN to inhibit Cu/Zn-SOD or H_2O_2 to inactivate both Cu/Zn-SOD and Fe-SOD, three types of SOD isozymes (Mn-SOD, Fe-SOD and Cu/Zn-SOD) were identified and the activity of each isozyme of the SOD was quantified (Fig. 1). When the activity of each isozyme of SOD was quantified on native PAGE gels by comparing the intensity of SOD activity in the leaf extracts relative to a known standard, PCB application caused a significant increase in the activation of all isozymes of SOD observed indicating all isoforms of SOD proved to be responsive to PCB stress. The chloroplastic location of Fe-SOD is related to the protection from oxidative stress occurring in this organelle (Donaue et al. 1997). The reduction of superoxide radical and the enhancement of hydrogen peroxide with PCB treatments (Table 1) could be ascribed to a rapid expression of Fe-SOD. Although the origin of Cu/Zn-SOD observed on activity gel is not known, the activity of the enzyme both in the cytosol and the chloroplast is likely to be induced by metabolic alterations con-

nected with PCB stress. In plants, CuZn-SOD is an isoform with the highest induction level in response to various stimuli (Mittler and Zilinskas 1994, Kaminaka et al., 1999), and the chloroplastic CuZn-SOD could contribute to O_2^- elimination in the chloroplast (Polle 1997). The Mn-SOD activity detected in this experiment was not superior to the activities of Fe-SOD and CuZn-SOD. Similar results have been observed in most plant species (Bowler et al. 1992). The lower activity might indicate that mitochondria where Mn-SOD isoforms could be related to O_2^- generation by the electron transport chain (Borsani et al. 2001) was less affected by PCB exposure. Although the roles of SOD isozymes in oxidative stress are not understood very well and the subcellular localization of CuZn-SOD was not investigated in this experiment, all the three isozymes measured in this study might contribute to the dismutation of superoxide radicals mainly with
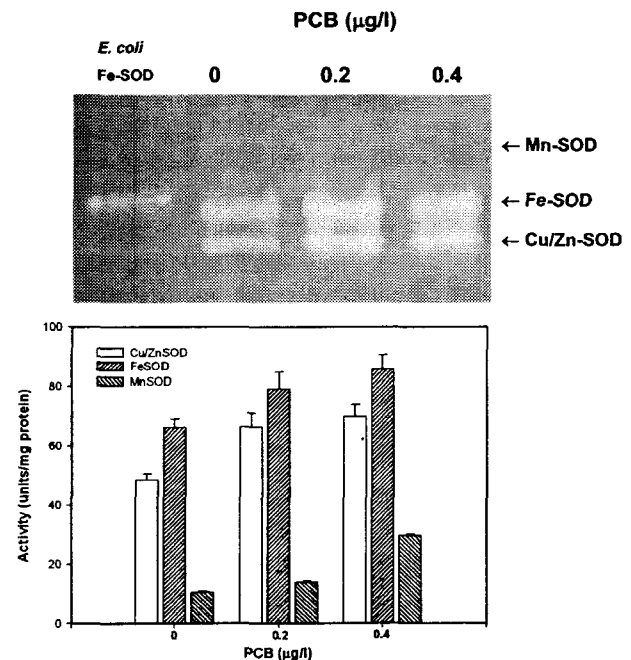
Fig. 1. Patterns and activities of isozymes of SOD in tomato leaves collected on day-5 after PCB treatment. Isozyme activities were determined on three separate extracts (replications) from seedlings and are reported relative to E. coli Fe-SOD on the same PAGE gel. Data are the mean ± SE.

Fe-SOD and Cu/Zn-SOD. However, it was not possible to draw conclusions about the cellular compartments which are more affected by the induced PCB stress.

The expressions of isozymes of POD and APX were also analyzed based on the band intensity of activity gel measured by analysis software (Figs. 2 and 3). Among four isozymes (POD-1, 2, 3 and 4) of POD identified on the activity gels, POD-1 and 2 were increased and POD-3 and 4 were decreased with PCB exposure as compared with control plants (Fig. 2). Although the enhanced activity of peroxidase with 0.4 $\mu g/l$ of PCB (Table 2) was probably due to the POD-1 and 2, the roles of POD isozymes were not known at this time. The relative distribution of peroxidase might be involved in the removal of H_2O_2 as well as lignification of tissues (Otter and Polle 1994) in response to PCB. Although the peroxidase reaction is unspecific towards the type of stress (Radotic *et al.* 2000), the capacity to synthesize peroxidases in leaves was used as a parameter for monitoring and mapping the defense of pollution (Keller 1974). The mechanism of peroxidase action in plants exposed to elevated concentrations of PCB has not been elucidated, nor has the evaluation of the phytotoxicity of PCB been performed completely either.

Three isozymes of APX were observed on the activity gel (Fig. 3). Based on the linescan data, all three isozymes of APX were increased with PCB exposure. The enhancement of total APX activity (Table 2) could be induced by the enhanced expression of all isozymes, and all the isozymes might contribute to the removal of PCB-induced H_2O_2. The dramatic increase of APX might be due to the localization of APX in cytosol and subsequent higher affinity for H_2O_2 (Asada 1992). Further, the high APX activity measured in this study suggest that APX in leaves may also be a key enzyme for the decomposition of H_2O_2 in PCB-induced oxidative stress.

The increased activities of SOD, GPX and APX implied that PCB application promoted the production of H_2O_2. The increased lipid peroxidation is probably due to the harmful effect of over production of H_2O_2 or its poisonous ROS derivatives particularly

PCB (µg/l)
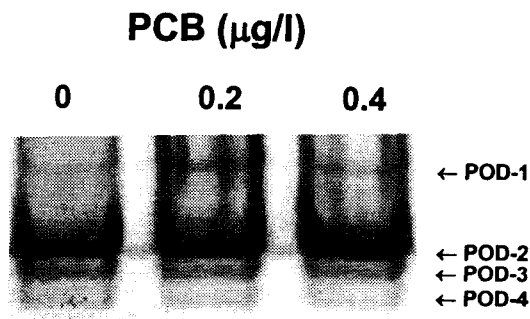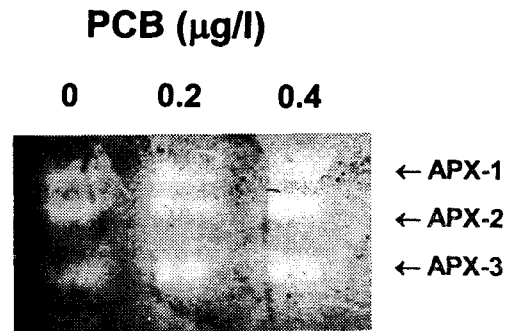

Fig. 3. Patterns of isozymes of a native PAGE for APX activities in leaf extracts from tomato seedlings collected on day-5 after PCB treatment.

in the chloroplast (Bowler *et al.* 1992) and insufficient activities of antioxidant enzymes. Excessive levels of ROS result in damage to the cell organelles including photosynthetic apparatus, ultimately leading to severe cellular damage and chlorosis of leaves. Once produced, O_2^- will rapidly dismutate to yield H_2O_2 and O_2 enzymatically or non-enzymatically. H_2O_2 itself is a powerful inhibitor of metabolism including carbon fixation (Kaiser 1976). Further, oxidation-reduction of metal ions by H_2O_2 and O_2^- through Haber-Weiss reaction produces the most toxic hydroxyl radical (· OH) (Imlay and Linn 1988, Halliwell and Gutteridge 1989). Therefore, the reduction of seedling growth observed after PCB exposure is probably due to the enhanced level of H_2O_2 and derivative ROS.

Our results show that tomato seedlings exhibit a well-defined activity of the enzymatic antioxidant system, which operates in seedlings subjected to PCB stress. The seedlings exposed to PCB-29 experience oxidative stress, and antioxidant enzymes are selectively activated. However, further researches on the specific distributions, identification and roles of the isozymes and the potential for ROS production in different organelles are required to explain the distinct regulatory mechanisms of antioxidant enzymes.

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PCB (µg/l)

Fig. 2. Patterns of isozymes of a native PAGE gel for POD activities of leaf extracts from tomato seedlings collected on day-5 after PCB treatment.

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