CORRELATION OF PCB TRANSFORMATION BY PLANT TISSUE CULTURES WITH THEIR MORPHOLOGY AND PEROXIDASE ACTIVITY CHANGES

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The ability of plant cells cultivated *in vitro* to metabolize polychlorinated biphenyls (PCBs) was correlated with the morphology of the cultures tested as models for phytoremediation studies. More differentiated cultures showed generally higher transformation capacity. The ability of plant cells to transform PCBs is connected to their viability in the presence of PCBs and their behaviour can be positively correlated with the production of intracellular and extracellular peroxidases. The cultures with high PCB-transforming activity proved to exhibit high peroxidase activity in the presence of PCBs while those with low ability to metabolize PCB showed a decrease of the enzyme activity in the presence of PCBs. Experiments with propylgallate were used to distinguish the ratio of involvement of peroxidases in PCB metabolism.

Key words: Polychlorinated biphenyls; Plant cells; Phytoremediation; Peroxidases; Inhibition; Biocatalysis; Oxidations; Xenobiotics.

Polychlorinated biphenyls (PCBs) are man-made compounds which were widely used in various industrial applications for many years due to their advantageous physico-chemical properties. Later on it was found that these compounds are toxic and nowadays PCBs remain among the most dangerous xenobiotics in the environment. The removal of PCBs by classical, *e.g.* physical methods is expensive, so cheaper remediation techniques like bioremediation seem to be promising technologies for the decontamination of polluted areas. Microorganisms are able to degrade xenobiotics under

aerobic and anaerobic conditions, and the microbial metabolism of PCBs has been an area of active research[1]. Little attention has been given to the attributes of plants in this regard. The use of plants to remove, contain or render harmless environmental contaminants is called phytoremediation. Phytoremediation is a relatively new concept which has several advantages over other commonly used remediation techniques[2,3]. It can be used for various types of contaminants, it is less disruptive to the environment, it has almost certain public acceptance and it avoids the need for excavation and soil transport. On the other hand, phytoremediation may take longer than physical, chemical or bacteriological methods, and the establishment of vegetation may be influenced by contaminant toxicity[4].

In practical application, phytoremediation has been used for *in situ* treatment of contaminated soils and sediments[5] and it has been shown to be effective in a number of full-scale and pilot studies. Nevertheless, there are still questions to be answered, and much basic and applied research is needed to make this technology a viable alternative across a wide range of sites.

For studying the fate of xenobiotics in plants *in vitro* techniques using plant cell, tissue and organ cultures have been applied[6–8]. Plant cells cultivated *in vitro* have many advantages as a model for phytoremediation studies. They grow at standard and defined laboratory conditions, the experiments are faster, not dependent on weather conditions and they require less analytical expenses. In our experiments we used plant tissue cultures of different morphology (callus cultures, shooty terratomas, hairy roots) to follow PCBs metabolism in plants. Previous studies demonstrated that plant cells are able to transform PCBs (ref.[9]). Hairy root cultures, transformed by *Agrobacterium rhizogenes*, have shown to be suitable model for phytoremediation studies[10]. In the present study we evaluated the ability of various morphologically different clones of two plant species, *Solanum aviculare* and *S. nigrum*, to transform PCBs and we compared the toxic effect of PCBs on the growth of these cultures. We have shown that the ability of plant cells to metabolize PCBs is strictly connected with their viability in the presence of PCBs and this behaviour can be positively correlated with the production of intracellular and extracellular peroxidases. The involvement of peroxidases in PCB metabolism is discussed.

EXPERIMENTAL

Plant Cell Culture

In vitro cultures of *Solanum aviculare* and *S. nigrum* were obtained from the Collection of Plant Tissue Cultures of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Cultures used and their morphological characteristics are listed in Table II. The selection includes amorphous nondifferentiated callus strains, together with cultures transformed by Ti or Ri plasmids of *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively. These cultures represent fully differentiated hairy root clones, strains forming embryoids or shooty terratomas, and undifferentiated cultures.

Polychlorinated Biphenyls

A standard commercial PCB mixture, Delor 103, containing 59 individual congeners substituted with 3–5 chlorines per biphenyl molecule was used. PCBs were added in methanol solution (1 ml) to give an initial PCB concentration of 2.5–5 mg in 100 ml of nutrient medium (25–50 ppm).

Cultivation

The plant cells were aseptically grown as submerged cultures in 250 ml Erlenmayer flasks containing 100 ml of nutrient medium[11]. For experiments, standard fresh weight (5 g FW) of inoculum in late exponential phase of growth (between 2–4 weeks old according to the strain tested) was used. Cells were incubated with PCBs (25.50 ppm) for 14 days in the dark. Dead cells (killed by boiling) and/or media without cells were used as controls[8]. At the end of incubation period, the living cells and also controls were heated at 90 °C for 25 min on a water bath.

Studies with inhibitor were carried out the same way as incubations with Delor 103, only propylgallate in concentration 0.1 mmol/l was added to selected flasks.

PCB Analysis

The analytical approach used in earlier experiments with microorganisms was modified for the use of plant biomass[8]. After homogenization and subsequent sonication, the contents of the flasks were extracted by 10 ml of hexane at 20 °C on a rotary shaker for 2 or 6 h, respectively. Following phase separation, the upper, hexane layer was sampled for GC analysis. Samples were analyzed using a Hewlett–Packard 5890 gas chromatograph with an electron capture detector and a fused silica capillary column (30 m, 0.20 mm i.d.) coated with 0.25 μm immobilized phase SE-54 and nitrogen as the carrier gas (flow rate 1 ml/min). The temperature programme was 50 °C for 1 min, then 25 °C/min until 280 °C, then isothermally. The amount of sample injected was 2 μl. Using GC analysis with EC detector, 22 of the 59 Delor congeners are assigned to peaks with area larger than 0.5% of the total area of all 59 individual chromatographic peaks[12]. For the calculation of the residual amount of PCBs of the above mentioned 22 chromatographic peaks were used (see Table I). These 22 congeners represent 80–90% of the total sample amount. Controls comprising of heat-killed cells were included to establish that observed changes in the content of congeners were dependent exclusively on the activity of living cells. Using standard conditions for the preparation of

samples for GC analysis, the accuracy of the results obtained was within 15%. Results were calculated from the residual amounts of each congener peak of the sample, comparing to the respective peaks of the controls, or using the value recommended by US EPA (Environmental Protection Agency) for expressing the total content of PCBs as a sum of recommended "indicator" congeners. For Delor 103 the following congeners (IUPAC nomenclature) were recommended: 28 (GC peak No. 7), 52 (peak No. 10) and 101 (peak No. 21) (EPA: US/EPA Methods 8089/8081).

TABLE I
Assignment of PCB congeners to peaks of Delor 103 analyzed on gas chromatography-EC detector

Peak No.	IUPAC No.	Substitution
1	5 + 8	2,3 + 2,4′
2	15 + 18	4,4′ + 2,2′,5
3	17	2,2′4
4	16 + 32	2,2′,3 + 2,4′6
5	26	2.3′,5
6	31	2,4′,5
7	28	2,4,4′
8	20 + 33 + 53	2,3,3′ + 2′,3,4 + 2,2′,5,6′
9	45	2,2′,3,6
10	52	2,2′,5′,5
11	49	2,2′,4,5′
12	47 + 75	2,2′,4,4′ + 2,4,4′,6
13	48	2,2′,4,5
14	44	2,2′,3,5′
15	37 + 42 + 59	3,4,4′ + 2,2′,3,4′ + 2,3,3′,6
16	41 + 64	2,2′,3,4 + 2,3,4′,6
17	96	2,2′,3,6,6′
18	74	2,4,4′,5
19	70	2,3′,4,5
20	66 + 88 + 95	2,3,4,4′ + 2,2′,3,4,6 + 2,2′3,5′,6
21	101	2,2′4,5,5′
22	77 + 110	3,3′,4,4′ + 2,3,3′,4′,6

Analysis of Peroxidases

To estimate the changes in peroxidase activity, additional cultivation vessels were incubated in parallels with the samples for estimation of PCB transformation. Flasks of the same inoculum in the same medium either did not contain Delor 103 (–D), or contained 2.5–5 mg of Delor 103 (+D) in 100 ml of nutrient medium.

Cell extracts were prepared by homogenization of 1 g frozen cell fresh weight by pestle and mortar with 1 ml of 0.1 M phosphate buffer pH 6.5 followed by centrifugation for 15 min. The total peroxidase activity in the cell extract and in the medium was measured by the chemiluminiscent method as was previously described[13,14] on a LUMAC 1500 apparatus from Celsis–Lumac Company (The Netherlands).

Electrophoresis

The isoenzyme pattern of peroxidases in the cell extracts and in the medium was analyzed by native electrophoresis in polyacrylamide gel after visualization of peroxidase isoenzymes with guaiacol (13 mmol/l) and hydrogen peroxide (5 mmol/l) in 0.1 mM phosphate buffer pH 6.5 for 15 min (ref.[9]).

RESULTS AND DISCUSSION

Reports on transformation of PCBs by plant cells in some cases document their good capacity to remove individual PCBs and their mixtures from the environment[7,9,10,15], even though a lack of information exists on PCB metabolism, products, enzymatic systems involved in PCB transformation and the toxicity of PCBs for plants. Recent reports of experiments with tissue cultures of different plant species cultivated *in vitro* were surveyed. These cultures did not differ in their morphology and only amorphous callus cultures were usually used throughout. Our study demonstrates the PCB removing abilities of several *in vitro* cultures of two plant species, *Solanum aviculare* and *S. nigrum*, differing in their morphology (Table II). Differentiated shooty terratomas, embryogenic or hairy root cultures often have similar morphologies as normal plants, they have wider metabolic capacity than callus cultures and they can be regenerated to normal plants.

Results summarized in Table II have shown that morphologically different clones of the same species are able to take up and transform PCBs with different efficiency. Within one species mostly differentiated cultures and those transformed by soil bacterium *Agrobacterium* exhibited higher efficiency to convert PCBs than non-differentiated and/or non-transformed cultures. Similar results were obtained by Harms and Kottutz[6] searching for the conversion of anthracene and phenanthrene by cell suspension, root culture and intact plants. They found no qualitative differences in metabolites formed, but they observed differences in converted amounts of initial

substances by cultures of different morphology. For this reason, when searching for a model system yielding the highest ability to metabolize PCBs and to allow effective study of the metabolism, enzymic systems and products formed, we screened and compared clones of the same species dif-

TABLE II
Metabolism of the PCB mixture Delor 103 by various cultures of *Solanum aviculare* and *S. nigrum* with different morphological characteristics, specification of the cultures and their growth in the presence (+D) and absence (–D) of Delor 103. Delor 103 was present in initial concentration 50 ppm, inoculum size was 5 g FW

Species + Culture	Morphology	Genetic transformation	FW (+D) g	FW (–D) g	PCB converted by plant cells %
Solanum aviculare					
AVC-7	hairy root	*A. rhizogenes*	3.2	5.4	0
KK1N	callus	no	7	29	12
KS1	callus	no	5.5	16.2	10
GK1	callus	no	5.4	20.3	20
AGS-3	callus	*A. tumefaciens*	5.9	25.2	25
AGC-1	callus	*A. tumefaciens*	13.4	7	40
C	callus	*A. tumefaciens*	10.3	22.4	25
AGR-1	callus	*A. tumefaciens*	5	20	28
AGC-3	hairy root	*A. rhizogenes*	8.4	10.9	40
AVR-1	shooty teratomas	*A. tumefaciens*	7.5	6.9	30
Solanum nigrum					
SNA-6	shooty teratomas	*A. tumefaciens*	5.8	6	0
SNC-7C	callus	*A. tumefaciens*	4.5	10.5	20
SNC-9O	hairy root	*A. rhizogenes*	28	33	45
SNC-7H	hairy root	*A. rhizogenes*	20.7	21.3	45
SNT-2	shooty teratomas	*A. tumefaciens*	9.2	12.3	45
SNT-4	shooty teratomas	*A. tumefaciens*	6.7	8.6	25

fering in morphology. As an example, comparison of the extent of transformation of selected 22 individual congeners of Delor 103 (ref.[12]) (Table I) by the clone *Solanum nigrum* SNC-90, which exhibited good transformation capacity, and by the cells of the clone SNC-7C, with low transformation capacity, is shown in Fig. 1. Original chromatograms obtained by GC analysis of the samples containing living and dead cells of SNC-7C and SNC-90 are documented in Fig. 2. It can be seen that the transformation of PCBs by the callus culture of SNC-7C mostly did not reach 20% (Fig. 1), while the hairy root culture SNC-90 of the same plant species removed PCBs with much higher efficiency.

Viability (expressed as the biomass fresh weight yield obtained from standard 5 g inoculum after 14 days cultivation in the presence of PCBs) (Table II) of the cultures with better PCB metabolizing potential was higher than viability of those which metabolized PCB with low efficiencies (AVC-7, KK1N, KS1, GK1, SNC-7C).

Parallely with the transformation of PCBs and the growth of plant biomass, the changes in total peroxidase activity under the influence of PCBs were followed. The lack of information currently available concerning the enzymic systems directly participating in the *in vivo* transformation (oxidation) of xenobiotic compounds in plants led us to study the possible role of peroxidases in metabolism of PCB detoxification.

Analytical studies of the products of the xenobiotic compounds have shown that plant cells possess the enzymatic capacity for initial oxidation

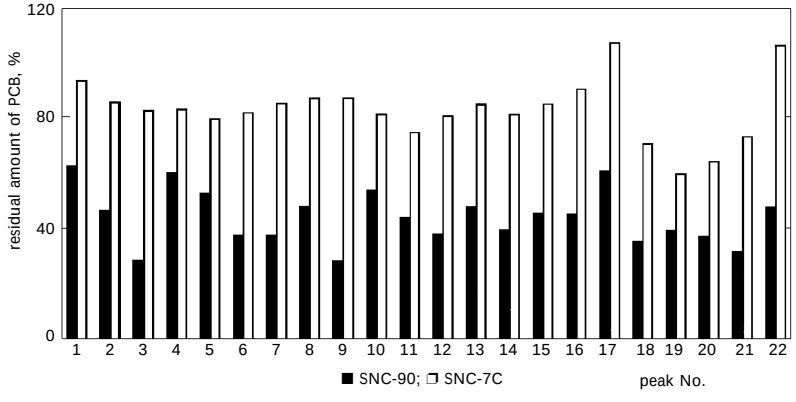

FIG. 1
Residual amounts of 22 individual congeners of PCB present in Delor 103 after incubation with cultures of *Solanum nigrum* SNC-90 exhibiting good transformation ability and SNC-7C with low ability to transform PCBs

and the formation of hydroxylated products[7], which are sometimes followed by glycosylation, polymerization, *etc.*[16]. The same type of initial oxidative reactions are characteristic for animal systems where mainly mixed-function oxidases with cytochrome P-450 are involved. Plants also possess similar mixed-function oxidase systems and, in addition to peroxidases and oxidases containing cytochrome P-450, dehydrogenases and other types of oxidizing enzymes have been identified in plant cells[17]. The exact functions of these enzymes, especially in PCB metabolism, are largely unexplored and it is still questionable whether the major enzymes participating in oxidative reactions of xenobiotics in plants are the same enzymes as in animal cells.

When analyzing the total intracellular and extracellular peroxidases, the level of peroxidase activity of the clones exhibiting high ability to metabolize PCB was comparable to or even higher than that of the controls incubated without PCBs (Fig. 3). Cultures unable to transform PCBs revealed the opposite, *i.e.* a decrease in peroxidase activity in the presence of PCBs (Fig. 4). Although it is suggested that a mixed-function oxidase system containing cytochrome P-450 plays an important role in the metabolism of

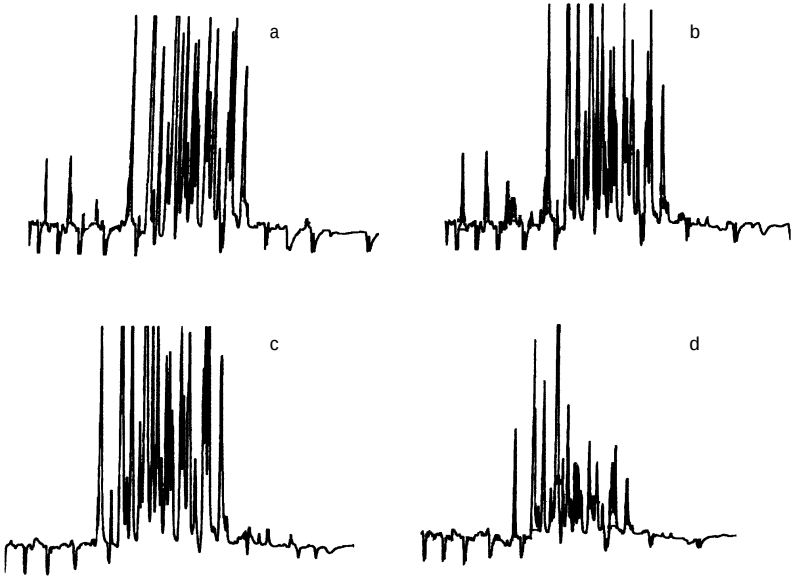

FIG. 2

GC chromatograms illustrating the metabolization of PCB mixture Delor 103 by two different *in vitro* cultured clones of *Solanum nigrum* in comparison with dead cell controls. a SNC 7C dead cells, b SNC 7C living cells, c SNC 90 dead cells, d SNC 90 living cells

PCBs and transformations of other xenobiotics[15] in plants, our results revealed a good correlation between PCB transformation and levels of peroxidase activity. There are many experimental data supporting the hypothesis of the involvement of peroxidases in oxidative transformations of xenobiotic compounds[18–20]. Sandermann[21] described that plant peroxidases have been identified to form electrophilic reactive intermediates from variety of xenobiotics which can further form bound residues and thus decrease the phytotoxicity of xenobiotics. Hypotheses of the possible functions of peroxidases in oxidative transformation reactions of certain organic compounds or of the participation of peroxidases in stressed detoxification metabolism[22] can be also further supported by the general properties of peroxidases as suggested by Stiborová and Anzenbacher[20]. They are present in large amounts in plant cells in contrast to low amounts of cytochrome P-450, they are localized in all parts of plant cells (cytochromes P-450 are located only in the microsomal fraction) and they have high affinities to

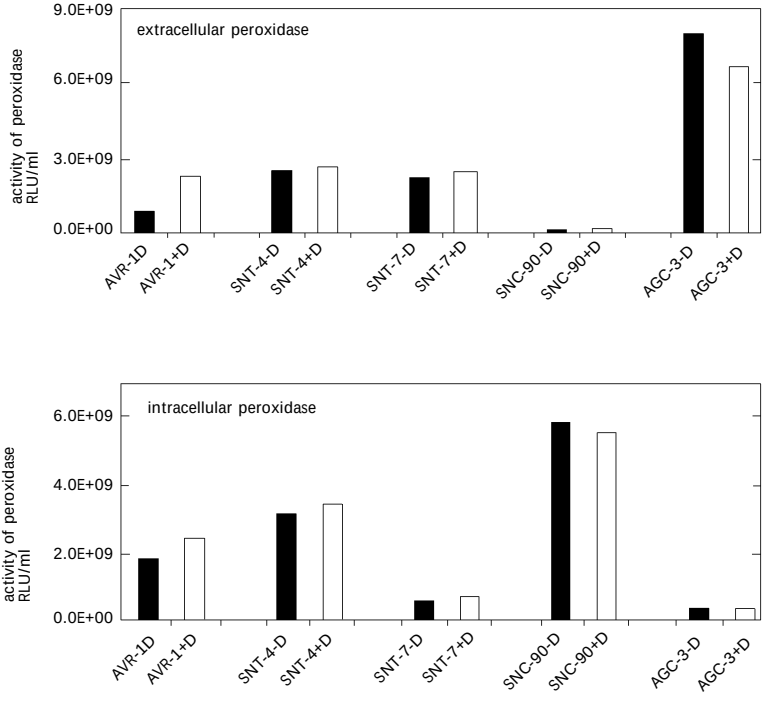

FIG. 3

Total extracellular and intracellular peroxidase activities of chosen cultures with good PCB-transformation abilities measured after incubation with (+D) and without (–D) Delor 103

exogenous substrates. Peroxidases are enzymes with wide substrate specifities participating *in vivo* in various important reactions. It was documented that plant peroxidases can catalyze N- or C-hydroxylation, N-sulfoxidation, N-acetylation, halogenation, dehalogenation or decarboxylation[17,23]. They are usually present in multiple isozymic forms which can be inducible by biotic or abiotic stress[22]. Formation of the new peroxidase isozymic forms in several clones with good transformation capacity which appeared as a consequence of PCBs presence was already shown[14].

To test the involvement of peroxidases in PCB metabolism we compared PCB-transformation capacity and peroxidase activities in presence of propylgallate. The clone SNC-90 of *Solanum nigrum* was chosen as a suitable plant model system for these studies. From Table II it can be seen that this clone is a fully differentiated hairy root culture which has exhibited good

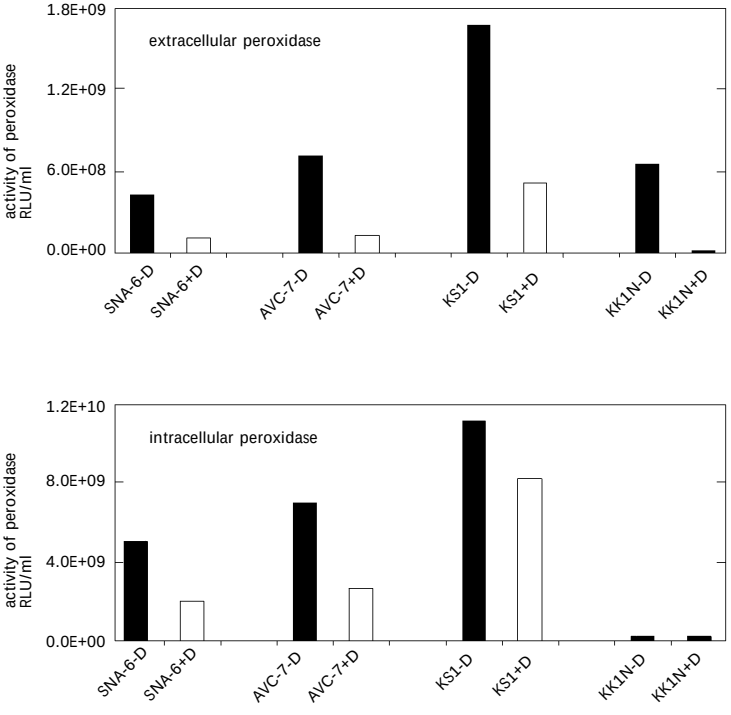

FIG. 4

Total extracellular and intracellular peroxidase activities of chosen cultures with low capacity to transform PCB after incubation with (+D) and without (–D) Delor 103

PCB-transformation capacity and satisfactory growth in the presence of PCB. Also, suitable incubation conditions of this culture have been already established[10]. Results obtained in experiments when propylgallate was used (Table III) showed a strong decrease in total extracellular and intracellular peroxidase activities after a 14 day incubation period. The inhibition of synthesis and activity of peroxidase isoenzymes by propylgallate is shown by native electrophoresis of the samples obtained after incubation with Delor 103 and with and without propylgallate (Fig. 5). The provision of this inhibitor also extremely decreased the PCB-transformation ability and the growth of plant cells of the clone SNC-90. On the other hand, Lee and Fletcher[15] reported slight effect of propylgallate on PCB transformation by

TABLE III
The effect of propylgallate on PCB transformation, cell growth and peroxidase activities (both extracellular and intracellular) of the clone SNC-90 of *Solanum nigrum*

Presence of propylgallate mmol/l	Cell growth, g		PCBs transformed[a] %	Residual POX activity[b], %	
	(+D)	(–D)		extracellular	intracellular
0.0	21	32.8	83	87	82
0.1	4.7	5.7	0	0.19	1.3

[a] The value of PCBs transformed by plant cells was calculated as a sum of "indicator" congeners (see Experimental); [b] residual peroxidase (POX) activities represent activities referred to the controls incubated without inhibitor and PCBs after 14 day incubation. Delor 103 was present in the concentration of 25 ppm

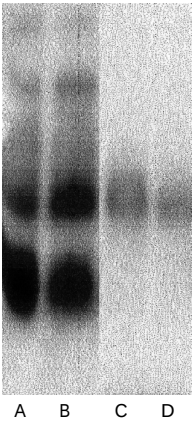

FIG. 5
Comparison of peroxidase isoenzyme pattern of the samples after incubation with PCBs and propylgallate (PG): A without Delor 103 and without PG, B with Delor 103 and without PG, C without Delor 103 and with PG, D with Delor 103 and with PG. Peroxidase isoenzymes were vizualized after 15 min, incubation with guaiacol (13 mmol/l) and hydrogen peroxide (5 mmol/l)

Paul's Scarlet rose callus culture. Their study compared the effect of various inhibitors of peroxidase and cytochrome P450 on PCB-transformation ability but this study did not include simultaneous analysis of enzyme activities and thus did not document the effect on enzyme stimulation or inhibition.

CONCLUSIONS

The data presented in this paper confirmed the ability of plant cells to metabolize PCBs to the same extent as many bacterial and fungal cells[24,25]. Plant tissue cultures can be used as a suitable model for studying the xenobiotic metabolism as was already published, but it is important to note that morphologically different cultures of the same species should be tested before making any conclusion due to their high variability and unequal metabolic activities. For this reason, any result obtained with only one or two *in vitro* cultures of the same plant species can not be generalized to the whole plant species or to a higher taxonomical unit.

Analyses of our data document the possible role of peroxidases in PCB metabolism. There may be a close connection between xenobiotic metabolism and the ability of plant cells to survive and grow under stress conditions, since both are affected by plant peroxidases. Above we discussed facts supporting the hypothesis[20] about the role of peroxidases in xenobiotic metabolism, and PCBs in particular[26]. In addition to phenomena already demonstrated, peroxidases also have other unique properties supporting their advantages over other enzyme systems in this respect. These general facts together with our results on PCB metabolism indicate, that peroxidases are involved in the metabolism of polychlorinated biphenyls by plant cells. For full understanding of the processes involved in PCB conversion by plants, it is crucial to prove the exact role of both mixed-function oxidase systems and to identify the reactions catalyzed by these enzymes.

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