Metabolism of selected polychlorobiphenyl congeners in plant cell cultures

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1. Introduction

Because of their extensive use, their high persistence and their toxicity, polychlorobiphenyls (PCBs) are industrial chemicals of environmental concern. They accumulate in food chains and are hazardous to humans. The effects and fate of PCBs in animals are well understood. They cause dermal toxicity, hepatoxicity, and are carcinogenic. With plants, the knowledge about the fate of PCBs is poor. Plants are able to take up polychlorobiphenyls by their roots from soils and from atmospheric deposition through their leaves. Previous studies showed that plants are able to metabolize PCBs by hydroxylation[1]. The metabolism rate depends on the chlorination grade and substitution pattern as well as on the plant species.

Here we report on the phytotoxicity, the metabolism and the characterization of metabolites of 2-chlorobiphenyl (PCB1), 2,2',5,5'-tetrachlorobiphenyl (PCB 52), and 3,3',4,4'-tetrachlorobiphenyl (PCB 77) in various plant cell cultures. For metabolism studies, ^{14}C-labelled PCB congeners were used.

2. Materials and Methods

Materials

Cell suspension cultures of 11 different plant species were obtained from the collection of the Institute of Plant Nutrition and Soil Science (FAL, Braunschweig, Germany).

Using an inoculum of 2 g cell material, the cultures were grown in 40 mL medium in 200 mL-Erlenmeyer flasks on a shaker (120 rpm) at 27°C. They grew in modified B5- or MS-media with the growth cycle optimized for each plant species. Cell cultures of the following plant species were used according to the conditions previously described[2]: *Glycine max* L. Merr. (soybean); *Trifolium repens* L. (clover); *Daucus carota* L. (carrot); *Lycopersicon esculentum* Mill. (tomato); Poaceae: *Hordeum vulgare* L. (barley); *Triticum aestivum* L. (wheat). Cell cultures of the following species were used according to the following culture conditions (growth cycle in days and the composition of the media): *Lupinus polyphyllus* L. (lupine) - 7 d; MS + 2 mg/L 2,4-D + 0.25 mg/L kinetine; *Lactuca sativa* L. (lettuce) -10 d; MS + 2 mg/L 2,4-D + 0.25 mg/L kinetine + 1 mg/L 6-Benzylaminopurin (BAP); *Brassica napus* L. (rape a.) - 8 d; MS + 2 mg/L 2,4-D; *Brassica napus* L. (rape b.) - 11 d ; MS + 2 mg/L 2,4-D; *Rosa spec.* var. Paul's Scarlett (rose) - 8 d; MS + 5 mg/L 2,4-D.

Chemicals

[U-^{14}C]-2-chlorobiphenyl (specific radioactivity 3.5×10^8 Bq/mmol) and [U-^{14}C]-2,2',5,5'-tetrachlorobiphenyl (specific radioactivity 4.0×10^8 Bq/mmol) were obtained from Sigma (Deisenhofen, Germany). [U-^{14}C]-3,3',4,4'-tetrachlorobiphenyl (specific radioactivity 12.2×10^8 Bq/mmol) were synthesized by 3). Hydroxylated PCBs 2-chloro-4-hydroxybiphenyl (RPM 1), 3-chloro-2-hydroxybiphenyl (RPM 2) and 2-chloro-5-hydroxybiphenyl (RPM 6) were purchased from Promochem (Wesel, Germany).

Phytotoxicity Tests

For phytotoxicity tests cell cultures were incubated with the congeners during the last 96 h (4 days) or over the total growth cycle. The concentration ranged between 1×10^{-4} M and 5×10^{-6} M for PCB 1, between 1×10^{-6} M and 5×10^{-9} M (=1ppb) for PCB 52 and between 5×10^{-6} M and 1×10^{-8} M for PCB 77. The concentration range used for the phytotoxicity tests are in the magnitude of the water solubility of the PCBs.

Metabolism Studies with ^{14}C-labelled PCBs and Hydrolysis

Metabolism studies were performed as previously described[2] with 1 ppm PCBs for the last 96 h of the growth cycle. Cells were harvested and extracted with dichloromethane/methanol. The medium, the cell extract and the nonextractable residues (after combustion in an oxidizer) were analyzed for radioactivity using liquid scintillation counting. The cell extract was seperated into two phases by adding dichoromethane and water. The upper (more polar) methanol/water phase contained the metabolites (conjugates) and was used for acid hydrolysis after concentration by evaporation. After addition of hydrochloric acid to give a 2N-HCl solution it was heated under reflux for 4 h.

Analysis

Thin layer chromatography (TLC) was performed on TLC-plates (Kieselgel 60 F_{254} Special, Riedel-de-Haën, Seelze, Germany). The TLC-plates were developed in Toluene : Ethylacetate (9 + 1) and were analyzed for radioactivity with an automatic TLC-Linear Analyzer (Tracemaster 20, Berthold, Wildbach, Germany). High pressure liquid chromatography (HPLC) separation was carried out using a 5-µm RP-18 column (ODS II Spherisorb, 250 × 4 mm, Techlab, Erkerode, Germany). PCB 77 was analyzed by HPLC with 5%-aqueous methanol (solvent A) and methanol (solvent B) in a gradient program starting with 50% solvent A for 5 minutes followed by a linear gradient to 90 % solvent B (constant for 1 minute) at a flow rate of 1 mL/min. Gaschromatography-mass spectrometry was performed as previously described.[2]

3. Results and Discussion

The investigated PCBs revealed no detectable phytotoxicity effects on the cell cultures at the tested concentrations. Only PCB 77 reduced growth of tomato at the highest tested concentration.

A screening of the ability of different plant cell cultures to metabolize PCB 1, PCB 52, and PCB 77 is summarized in Table 1. In accordance with analytical procedure, the metabolism rates were calculated from the amount of radioactivity measured in the methanol/water phase.

Table 1. Metabolism rate of different PCB congeners in various cell cultures

	Metabolism rate[a] of various plant species										
	Fabaceae			Apiaceae	Asteraceae	Solanaceae	Brassicaceae		Rosaceae	Poaceae	
	GM[b]	TR	LP	DC	LS	LE	BNa.	BNb.	PSR	HV	TA
PCB 77	-	-	-	-	+	+	-	-	++	-	-
PCB 1	+++			+							++
PCB 52				-							+++

[a]Rate of metabolism: - = no metabolism; + = metabolism rate < 15 %; ++ = metabolism rate 15 to 30%; +++ = metabolism rate 30 - 100 %; no sign= not investigated.

[b]GM: *Glycine max* L. Merr.; TR: *Trifolium repens* L.; Lupo: *Lupinus polyphyllus* L.; DC: *Daucus carota* L.; LS: *Lactuca sativa* L.; LE: *Lycopersicon esculentum* Mill.; BNa.: *Brassica napus* L.; BNb.: *Brassica napus* L.; PSR: Paul's Scarlett Rose; HV: *Hordeum vulgare* L.; TA: *Triticum aestivum* L.

The tetrachlorinated PCB 77, which has a low water solubility (1.88×10^{-9} M), was metabolized by only three of the tested cultures. These were tomato (LE), lettuce (LS), and to the greatest extend, Paul's Scarlett Rose (PSR). Generally PSR seems to exhibit a great ability to metabolize a number of polychlorobiphenyl congeners[1], e. g. 2,2'-dichlorobiphenyl (PCB 4) by 100 %, 2,4'-dichlorobiphenyl by 90 % as well as 2,2',5-trichlorobiphenyl, and 2,4',5-trichlorobiphenyl. All other tested cultures did not metabolize PCB 77.

Recent studies[2] with different PCB congeners showed that the metabolism was strongly dependent on the plant species. Two Fabaceae species in particular exhibited high capacity for metabolizing different congeners. Furthermore it was shown that a lower chlorination grade is associated with higher metabolism rates, and that substitution and molecular configuration play an important role in PCB metabolism in plants. In our case soybean cultures metabolized PCB 1 to a high degree, but the same culture, like the other tested Fabaceae did not metabolize PCB 77. The same is true for wheat cultures which metabolize PCB 1 and PCB 52 but not PCB 77. The para-position of the chlorobiphenyl seems to be attacked preferentially[2]. 2-chlorobiphenyl (PCB 1) and 2,2',5,5'-tetrachlorobiphenyl (PCB 52) have free para-positions and thus are better metabolized in contrast to 3,3',4,4'-tetrachlorobiphenyl (PCB 77) where the para-positions are substituted by chlorine. This again demonstrates that the metabolism of PCBs in plants is dependant on the plant species and the physico-chemical properties of the PCB molecule.

For further analysis, the cell material from experiments which exhibited sufficient turnover rates was extracted with organic solvents. The distribution in different fractions, as medium, polar and less polar phases and nonextractable residue is given in Table 2.

Table 2.: Distribution of radioactivity [%] in different fractions after application of ^{14}C-PCB 1, ^{14}C-PCB 52 and ^{14}C-PCB 77

Fraction	PCB 1 GM[a]	PCB 52 TA[b]	PCB 77 LE[a]	PCB 77 LS[a]	PCB 77 PSR[b]
Medium	**7**	**7**	**13**	**< 1**	**6**
Cell extract	**58**	**69**	**81**	**95**	**81**
Methanol/water phase	36	57	2	8	18
Dichloromethane phase	22	12	79	87	63
Nonextractable residue	**10**	**8**	**1**	**1**	**2**
Recovery	**75**	**84**	**95**	**97**	**89**

[a]mean values of three parallels; [b] mean values of two parallels;

PCB 1= 2-chlorobiphenyl; PCB 52 = 2,2',5,5'-tetrachlorobiphenyl; PCB 77 = 3,3',4,4'-tetrachlorobiphenyl

The residual radioactivity in the media indicates that on average more than 90 % of applied congeners are taken up by the plant cells. For those cultures which exhibited high metabolism rates such as soybean for PCB 1 and wheat for PCB 52, most of the radioactivity was in the polar phase and noteworthy amounts were detected in the nonextractable residue fraction. In contrast, most of radioactivity of PCB 77 was detectable in the less polar dichloromethane phase in the form of parent compound. Only a little radioactivity was detected in the bound residue fraction. PSR was the only plant culture which showed noteworthy metabolism rates.

Analysis of the methanol/water phase (more polar) gave several polar metabolites that yielded a number of products after hydrolysis with HCl. Based on co-chromatography with authenic substances, these products were assumed to be hydroxylated PCBs.

For soybean, the GC-MS analysis after incubation of ^{14}C-PCB 1 resulted in the detection of six metabolites with masses 204 [M^+]. These are attributed to monohydroxy metabolites. This was confirmed by the fragments of m/z 168 [M^+ -HCl] and of m/z 139 [M^+ -HCl-HCO]. Furthermore, the GC-MS spectra showed one metabolite with mass 220 [M^+] which is attributed to a dihydroxy PCB. Expected fragments of dihydroxylated PCB 1 of 192 [M^+-CO], 185 [M^+-Cl], and 177 [M^+-Cl-CO]

were also detected. The loss of Cl/HCl by fragmentation of hydroxylated PCBs in MS analysis is known from literature[5].

The HPLC analysis of the soybean extract showed three major peaks for monohydroxylated metabolites and one for a dihydroxylated metabolite. Two of the monohydroxylated metabolites can be tentatively identified as 2-chloro-5-hydroxybiphenyl and 3-chloro-2-hydroxybiphenyl based on co-chromatography in HPLC and GC-MS. The latter compound might be metabolized by an NIH-Shift, which was observed to occur in the PCB metabolism in rats[6].

Accordingly the polar metabolites of PCB 52 were analyzed. The GC-MS resulted in four mono-hydroxylated (m/z = 308 [M^+]) and three dihydroxylated (m/z = 324 [M^+]) metabolites. A HPLC analysis showed three major peaks. It was not possible to determine the position of the hydroxy group in the molecule because there are no reference substances available.

The same methods are used for analysing PCB 77 metabolites. Figure 1. demonstrates the distribution of radioactivity on TLC of the hydrolyzed methanol/water phase of tomato cell cultures.

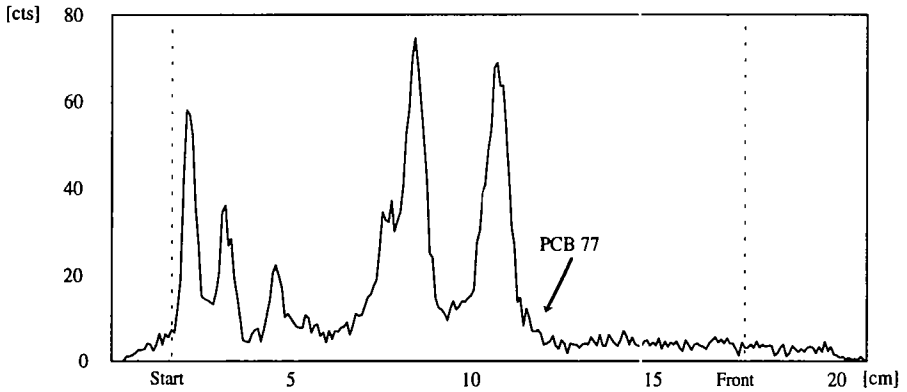

Figure 1: TLC in Toluene:Ethylacetate (9+1) of hydrolyzed methanol/water phase of PCB 77 metabolism in tomato

Figure 1 shows a trace from an analysis where at least 6 polar metabolites of the PCB 77 with retention factors 0.01, 0.07, 0.16, 0.36, 0.41, and 0.55 were detected. The metabolites are more polar than the parent PCB 77 compound which has a retention factor 0.64.

A high pressure liquid chromatogram of PCB 77 metabolites in tomato is shown in Figure 2.

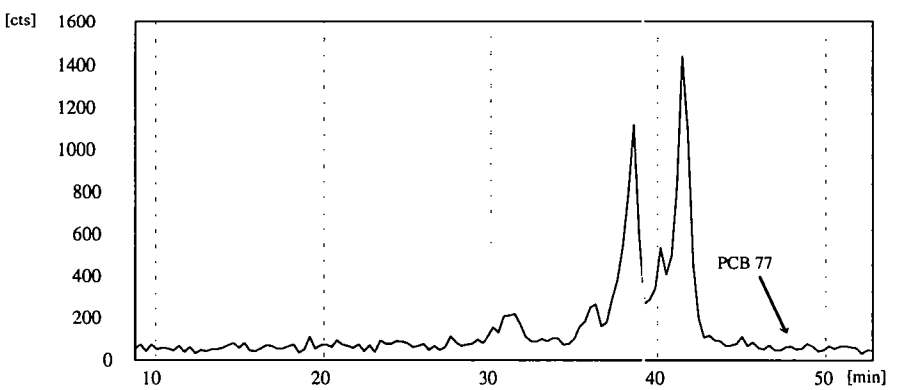

Fig. 2: HPLC of ^{14}C-PCB 77 metabolites (hydrolyzed methanol/water phase) in tomato cell cultures

The HPLC chromatogram of the hydrolyzed methanol/water phase shows at least 5 metabolites which are more polar than the PCB 77 with a retention time of 47.5 min.
The GC-MS analysis of PCB 77 metabolites remains to be carried out.

4. References

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