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Monitoring toxicity, DNA damage, and somatic mutations in tobacco plants growing in soil heavily polluted with polychlorinated biphenyls

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Abstract

Heterozygous tobacco (*Nicotiana tabacum* var. *xanthi*) plants were cultivated in soil from a dump site highly polluted with polychlorinated biphenyls (PCBs) at Lhenice in South Bohemia, Czech Republic. The total amount of PCBs in the polluted soil, measured by gas chromatography varied from 165 to 265 mg kg^{-1} of soil. In tobacco plants cultivated for 8 weeks in the polluted soil the amount of PCB in the leaves varied from 11 to 28 and in the roots from 104 to 308 mg kg^{-1} dry mass. The average leaf area of tobacco plants growing in the PCB-polluted soil was significantly reduced and the DNA damage in leaf nuclei, measured by the comet assay, was slightly but significantly increased compared with controls. The tobacco plants with increased DNA damage showed reduced growth and had distorted leaves. No increase in the frequency of somatic mutations was detected in tobacco plants growing in the PCB-polluted soil.

Keywords: Comet assay; Single-cell gel electrophoresis; Gas chromatography; *Nicotiana tabacum*

1. Introduction

Plants are exposed to various types of environmental agent, either deliberately as in the case of agricultural pesticides and plant-growth regulators, or accidentally as compounds present in polluted air, soil or water.

Polychlorinated biphenyls (PCBs) have been used as coolants and lubricants in transformers, capacitors, and other electrical equipment because they are highly resistant to high temperatures, and are good insulators. They belong to the group of persistent environmental pollutants, which can be found (at least in low quantities) in almost every compartment of terrestrial and aquatic ecosystems. The mechanisms by which PCBs exert their adverse effects on organisms are not fully understood and data on their genotoxic properties are still controversial [1,2].

In the work presented here, tobacco plants (*Nicotiana tabacum* var. *xanthi*) were cultivated for 2–8 weeks in soil from a dump site polluted with PCBs (locality Lhenice in South Bohemia, Czech Republic) and in

Abbreviations: PCBs, polychlorinated biphenyls; TM, tail moment

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control garden soil. We have (1) assessed the toxic effects of the soil samples by measuring the tobacco leaf area, (2) evaluated induced DNA damage in leaf nuclei by the comet assay, and (3) scored the frequency of somatic mutations in leaves. In order to evaluate the possible adverse effects of poor nutrient values of the PCB-polluted soil, the tobacco plants were watered in parallel pots with distilled water or with 50% Hoagland's solution. The amount of PCBs in the soil and in the tobacco leaves and roots was determined by gas chromatography with electron-capture detection.

2. Material and methods

2.1. Chemicals and media

Ethyl methanesulphonate (EMS, CAS No. 62-50-0), maleic hydrazide (MH, CAS No. 123-33-1) and reagents for electrophoresis were purchased from Sigma Chemical Co., St. Louis, MO, normal and low melting-point agarose from Roth, Karlsruhe, Germany. Other reagents for the determination of the content of PCBs in the soil and in the tobacco plants were from Fluka, Germany and PCB standards were from Dr. Ehrenstorfer Co., Germany.

2.2. PCB-polluted soil

PCB-contaminated soil was taken from a protected dump site in Lhenice, South Bohemia, Czech Republic. The soil was originally collected from places with high concentrations of PCBs, mostly from factories using PCBs for the production of incombustible materials, e.g. dyes, transformers or tar. The PCBs for these factories (mostly the commercial PCB mixture Delor 103) were produced by Chemko Strážské until 1984. The area of the dump site is about 500 m^2 and contains approximately 250 t of contaminated soil. The PCB content varied between 5 and 400 mg kg^{-1} soil. The PCB content in the control soil and in control plants was so low that it could not be detected.

2.3. Tobacco growth, treatment conditions, measurement of the leaf area and the frequency of somatic mutations

Seeds of the double heterozygous *N. tabacum* var. *xanthi* ($a_1^+/a_1; a_2^+/a_2$) plants [3] were germinated under sterile conditions in plastic vented containers that contained 50 ml of solid growth medium. At the stage of three to four true leaves, the plants were transferred to plastic pots with the test soil. Ten plants in pots *per* variant were cultivated in a cultivation room with artificial light with an 18-h photoperiod at 22–26 °C.

The leaf area of one leaf per plant, thus 10 leaves per variant, was measured by a planimeter and expressed in square centimetre. The measurements started after 2 weeks with the first newly formed leaves after planting the seedlings into the polluted soil. After 3, 4, 6 and 8 weeks the area of the subsequent leaves was measured.

Three main types of mutagenic event were scored on the greenish-yellow leaves of the heterozygous tobacco plants cultivated on the test soil: (1) dark green, (2) yellow and (3) green/yellow twin sectors [3]. The somatic mutations were scored under a stereomicroscope.

2.4. DNA-damage studies

The preparation of agarose microscope slides with isolated nuclei, the DNA unwinding, and the conditions of electrophoresis and ethidium bromide staining in the comet assay were as previously described [4,5]. For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A computerized image-analysis system (Komet Version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed. The tail moment parameter (integrated value of tail DNA density multiplied by the migration distance/100) was used as a measure of the DNA damage. For DNA-damage studies, three leaves were taken from different plants of each treatment variant, and from each leaf two comet slides were prepared. In total, 150 nuclei were analyzed *per* variant.

2.5. Determination of the content of PCB in the soil and in tobacco plants

Aliquots (1 g) of soil samples that had been air-dried overnight and sieved (1-mm mesh), and aliquots (5 g) of harvested and air-dried tobacco leaves and roots, homogenized in liquid nitrogen, were extracted with hexane for 4 h. The extracts were concentrated to 1 ml by a nitrogen flow, purified on a Florisil column, and diluted with hexane to the same volume as was used for the extraction. These extracts were analyzed using a Hewlett-Packard 5890 gas chromatograph with an electron-capture detector and a fused silica capillary column (30 m, 0.2 mm inner diameter) coated with 0.25 μm immobilised phase SE-54 with nitrogen as carrier gas (flow rate 1 ml min^{-1}). The temperature program was as follows: 50 °C for 1 min, followed by an increase at a rate of 25 °C min^{-1} until the temperature was 280 °C and then maintained at this temperature. The injection volume was 2 μl. Results were calculated from the residual amounts of congener peaks present in each sample. For the evaluation of the experiments, the US EPA method 8089/8081 for expressing the total content of PCBs as a sum of recommended indicator congeners (PCB 77, 101, 118, 138, 153, 180) was applied [6,7].

2.6. Statistical analysis

Data were analyzed using the statistical and graphical functions of SigmaPlot 8.0 and SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). The median tail moment values were used in a one-way analysis of variance test. If a significant F-value of $P < 0.05$ was obtained, a Dunnett's multiple comparison versus the con-

trol group analysis was conducted. Differences between two groups were statistically evaluated by the paired *t*-test.

3. Results

3.1. Toxic, mutagenic, and DNA-damaging effects in tobacco plants cultivated in soil with high levels of PCBs

Two independent experiments were conducted. As the content of PCBs in the polluted soil differed in both experiments, the data could not be combined.

3.1.1. Experiment 1

Data after 4, 6 and 8 weeks cultivation of tobacco plants in PCB-polluted and control soil are presented in Table 1. The averaged leaf area of plants cultivated in PCB-polluted soil for 8 weeks ($4.9 \pm 1.2\,cm^2$) is significantly ($P < 0.05$) lower than the leaf area of plants cultivated in control soil ($98.9 \pm 6.2\,cm^2$).

An increased frequency of somatic mutations would provide evidence of the genotoxic effects of the PCB-polluted soil. After 8 weeks of cultivation of the heterozygous tobacco plants, the mean frequency (±S.E.) of mutations in plants growing in the non-polluted soil was very low (1.8 ± 0.23). Nevertheless it was significantly ($P < 0.05$) higher than the mutation frequency in tobacco plants growing in PCB-polluted soil (0.3 ± 0.2 mutations *per* leaf scored). This may be explained by the very small size of the leaves of plants growing in polluted soil, which prevents the detection of small mutated sectors. However, the DNA damage, expressed by the TM value of $10.0 \pm 1.4\,\mu m$ was in leaf nuclei of plants cultivated for 8 weeks in PCB-polluted soil significantly ($P < 0.05$) higher than in nuclei from control plants ($2.3 \pm 0.4\,\mu m$). For comparison, the herbicide and plant-growth regulator maleic hydrazide induced in tobacco 37.8 ± 3.2 mutant sectors *per* leaf and the alkylating agent EMS induced DNA damage expressed by a TM value of $45.2 \pm 2.6\,\mu m$.

The PCB content in the polluted soil in experiment 1 reached a value of $265.5 \pm 12\,mg\,kg^{-1}$ soil. After an 8-week cultivation of tobacco plants on the soil the amount of PCBs decreased by 13%. The amount of PCBs in the leaves was 28.8 ± 12 and in the roots $104.5 \pm 8\,mg\,kg^{-1}$ dry mass (upper part of Table 3).

3.1.2. Experiment 2

We were interested to know if the inhibition of plant growth and the increase of DNA damage in tobacco plants growing on the PCB-polluted soil as demonstrated in experiment 1, was not affected also by the poor nutrient-values of the PCB-polluted soil. Thus the tobacco plants were watered in parallel pots with distilled water or with a 50% Hoagland's solution. The averaged leaf-area ($9.3 \pm 0.8\,cm^2$) after 8 weeks of cultivation of the plants in PCB-contaminated soil, watered with distilled water was significantly ($P < 0.05$) smaller than the leaf area of plants watered with a 50% Hoagland's solution ($21.2 \pm 2.6\,cm^2$). In tobacco plants cultivated in control soil the value of averaged leaf-area significantly ($P < 0.05$) increased to $65.5 \pm 4.6\,cm^2$ (Table 2).

After 8 weeks of cultivation of the heterozygous tobacco plants, the mean number (±S.E.) of mutant sectors *per* leaf in tobacco plants growing in PCB-polluted soils (0.5 ± 0.4) was slightly lower than in the negative controls (1.0 ± 0.3). The results of monitoring DNA damage in leaf nuclei of tobacco plants growing in the PCB-polluted and control soils are presented in the last column of Table 2. Although the DNA dam-

Table 1
Average leaf area, number of mutant sectors *per* leaf, and DNA damage (expressed as tail moment) in tobacco plants (*Nicotiana tabacum* var. *xanthi*) cultivated for 4–8 weeks on soil polluted with polychlorinated biphenyls (PCBs soil) or on control soil

Substrate	Cultivation (weeks)	Average leaf area ($cm^2 \pm$ S.E.)	Number of mutant sectors *per* leaf (±S.E.)	Tail moment ($\mu m \pm$ S.E.)
PCBs soil	4	3.5 ± 2.1	0.3 ± 0.1	3.8 ± 0.6
Control	4	42.4. ± 6.6	0.4 ± 0.2	1.2 ± 0.2
PCBs soil	6	5.8 ± 1.0	0.3 ± 0.2	2.0 ± 1.2
Control	6	78.7. ± 5.1	1.0 ± 0.2	1.6 ± 0.2
PCBs soil	8	4.9 ± 1.2	0.3 ± 0.2	10.0 ± 1.4
Control	8	98.9 ± 6.2	1.8 ± 0.2	2.3 ± 0.4
MH[a].	4	36.7 ± 2.1	37.8 ± 3.2	
EMS[b]	2			45.2 ± 2.6

[a] MH: tobacco plants treated with 0.05 mM maleic hydrazide.
[b] EMS: tobacco plants treated with 0.04 mM ethyl methanesulphonate.

Table 2
Average leaf area, number of mutant sectors *per* leaf, and DNA damage (expressed as tail moment) in tobacco plants (*N. tabacum* var. *xanthi*) cultivated for 2–8 weeks on soil polluted with polychlorinated biphenyls (PCBs soil) or on control soil

Substrate	Cultivation (weeks)	Average leaf area ($cm^2 \pm$ S.E.)	Number of mutant sectors *per* leaf (±S.E.)	Tail moment (μm ±S.E.)
PBCs soil (A)	2	9.5 ± 1.1	0.4 ± 0.1	1.6 ± 0.6
PBCs soil (B)	2	13.7 ± 1.6	0.4 ± 0.2	0.9 ± 0.2
Control (A)	2	31.1 ± 3.4	0.7 ± 0.2	0.8 ± 0.1
PBCs soil (A)	3	13.7 ± 1.7	0.4 ± 0.2	0.8 ± 0.1
PBCs soil (B)	3	22.1 ± 2.9	0.5 ± 0.3	0.5 ± 0.1
Control (A)	3	40.2 ± 3.7	1.3 ± 0.2	0.5 ± 0.1
PBCs soil (A)	4	8.4 ± 1.0	0.3 ± 0.2	3.5 ± 1.2
PBCs soil (B)	4	16.0 ± 1.1	0.4 ± 0.2	1.0 ± 0.2
Control (A)	4	54.2 ± 3.5	0.8 ± 0.4	1.2 ± 0.6
PBCs soil (A)	6	7.8 ± 1.2	0.4 ± 0.2	13.0 ± 1.4
PBCs soil (B)	6	18.9 ± 2.2	0.5 ± 0.2	4.6 ± 0.4
Control (A)	6	67.8 ± 4.6	1.0 ± 0.3	0.8 ± 0.4
PBCs soil (A)	8	9.3 ± 0.8	0.5 ± 0.4	11.1 ± 1.4
PBCs soil (B)	8	21.2 ± 2.6	0.5 ± 0.5	4.5 ± 0.4
Control (A)	8	65.5 ± 4.6	1.0 ± 0.3	1.6 ± 0.3

A: watered with distilled water, B: watered with 50% Hoagland's solution.

Table 3
Content of polychlorinated biphenyls (PCBs) in soil, and in leaves and roots of tobacco plants (*N. tabacum* var. *xanthi*) cultivated on PCB-polluted soil for 8 weeks

	Content of PCBs ($mg\,kg^{-1}$ soil)		Content of PCBs after 8 weeks of cultivation ($mg\,kg^{-1}$ dry mass)	
	Start of experiment	After 8 weeks	Leaves	Roots
Experiment 1				
Watered with distilled water	265.5 ± 12	233.3 ± 54	28.8 ± 12	104.5 ± 8
Experiment 2				
Watered with 50% Hoagland's solution	164.4 ± 15	120.8 ± 12	10.9 ± 5	271.1 ± 8
Watered with distilled water		153.0 ± 8	28.4 ± 4	307.9 ± 12

The results are means of triplicate experiments ± S.D.

age after 6 and 8 weeks cultivation of tobacco plants in the polluted soil was low (TM ± S.E, 13.0 ± 1.4 and 11.1 ± 1.4 μm, respectively) these values were significantly ($P < 0.05$) higher than the TM values in leaf nuclei isolated from tobacco plants cultivated in control soil (0.8 ± 0.4 and 1.6 ± 0.35 μm, respectively). The data in Table 2 demonstrate that the increased toxicity and the DNA damage in plants cultivated on PCB-polluted soil is not caused only by the PCBs, but probably also by the poor nutrient condition of the test soil, as watering the plants with a 50% Hoagland's solutions increased the leaf area and decreased the amount of DNA damage.

The content of PCBs measured by gas chromatography in the polluted soil at the start of experiment 2 (lower part of Table 3) was 164.4 ± 15 $mg\,kg^{-1}$ soil and its decrease during an 8-week period was dependent on the type of watering of the plants: with 50% Hoagland's solution the amount of PCBs decreased by 27%, whereas this was only 7% during watering with distilled water. The PCB content after 8 weeks of cultivation on PCB-polluted soil was significantly ($P < 0.05$) lower in the leaves than in the roots. The PCB content in the control soil and plants was undetectable.

4. Discussion

PCBs were, and in some countries still are, released into the environment during their manufacture, use, and disposal and from accidental spills and leaks. PCBs do not readily break down in the environment and thus may remain there for a long period of time [2]. Their accumulation in soils can become dangerous to all kinds of organisms, including plants [8]. However, there is an

increasing recognition that elevated contaminant levels by themselves are not necessarily indicative of actually occurring adverse effects. The most important factor for understanding the possible adverse effects of pollutants including PCBs is their actual accumulation in organisms. Some PCB congeners are slowly degraded in the environment and can enter the food chain. Several agencies have categorized PCBs as animal carcinogens; however, studies of workers exposed to high doses of PCBs have not demonstrated an increased cancer risk [1]. As far as results were obtained using the comet assay, planar PCB77 did not induce DNA damage in human lymphocytes or in brown trout [9,10]. None of the tested individual PCB congeners and PCB mixtures induced a significant increase of DNA damage in nuclei of human sperm in the neutral comet assay [11].

We have applied the alkaline protocol of the single-cell gel electrophoresis or comet assay [12,13] as a method for detecting induced DNA damage in tobacco plants growing on PCB-polluted soil. Although this technique has been primarily applied to animal cells, the use of the comet assay with plant tissues [14–16] significantly extends the utility of plants in basic and applied studies in environmental mutagenesis.

The data reported in this presentation demonstrate that the growth of tobacco plants (expressed by the average leaf area) on PCB-polluted soil is strongly inhibited compared with the growth of plants on control soil. The tobacco plants with slightly, but significantly increased DNA damage were severely injured (reduced growth, distorted and brownish leaves). Many of the comet images from these distorted leaves were of the "hedgehog" type (large fan-like tail and small heads). This may be associated with necrotic or apoptotic DNA fragmentation [5]. No increase in the frequency of somatic mutations was detected in tobacco plants growing on PCB-polluted soil. The PCB-induced toxicity, leading to a strong inhibition of leaf growth, could have inhibited the division of induced mutated cells, so that mutated sectors could not be detected.

As illustrated in Table 3, the roots accumulated about 3.6–25-fold more PCBs than leaves. However, for studying DNA damage by the comet assay the roots could not be used, as the number of readable nuclei was very limited.

When studying the toxic and genotoxic effects of polluted soil on plants, the composition of the soil and the pollutant of interest have to be taken into account. Such factors as the nutrient content of the soil, its pH, and microbial parameters may influence the results observed [17,18]. Our results demonstrate that adding nutrients to the PCB-polluted soil by watering with a Hoagland's solution markedly decreased its toxic and DNA-damaging effects.

The question has been raised whether the comet assay is a useful method for monitoring genotoxic effects of environmental pollutants in plants growing *in situ.* As was demonstrated here, a slight but significant increase in DNA damage was associated first with strongly toxic effects (growth inhibition, brownish and distorted leaves). Similar results were obtained when monitoring the toxic and genotoxic effects in tobacco and potato plants growing on soil heavily polluted with heavy metals [19]. Thus the comet assay probably does not represent a sensitive method for monitoring environmental pollutants using plants growing *in situ*.

In conclusion, the average leaf area of tobacco plants growing in PCB-polluted soil was significantly reduced, and the DNA damage, measured by the comet assay in leaf nuclei, was slightly but significantly increased. No increase in the frequency of somatic mutations was detected.

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