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Environmental Contamination and Toxicology

1,2,4-Trichlorobenzene Induction of Chromosomal Aberrations and Cell Division of Root-Tip Cells in *Vicia faba* Seedlings

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In the last several decades, halogenated aromatic hydrocarbons have been used extensively as intermediates in the chemical industry and agriculture, and are being introduced into the environment (Kong et al. 1998). Since some of these chemicals are carcinogenic, mutagenic and have long residence time in the environment, 25 species of halogeno-benzenes (HBs) were included in the 129 priority control pollutants listed in the US EPA (Gan et al. 2000). Due to their hydrophobicity and high hydrophobic partition coefficient (K_{ow}) in octhanol/water, HBs are easily accumulated in living organisms and have deleterious effect on DNA molecules directly or indirectly. HBs toxicity in many nontolerant organisms was reported to be associated with the disturbance of mitosis, induction of chromosomal aberrations and micronuclei formation and inhibition of some enzymes activity (Zapata-Gayon et al. 1982; Zhou 1986; Xia 2001). The above investigation is well reported and mainly focused on pesticides, fungicides, radiation and heavy metals stress (William et al. 1999; Grant et al. 1998; Kong et al. 1999; Shahin et al. 1991; Duan et al. 1995; de Kergommesaux 1983); whilst cytological research on the poisoning effects of HBs on crops has scarcely been reported. In order to further understand the cytotoxic effects of 1,2,4-trichlorobenzene (TCB) – a typical kind of HBs in crops, the effects of different concentrations of TCB on cell division, chromosomal aberrations and root growth of *V faba* seedlings have been studied in this paper.

MATERIALS AND METHODS

Well developed seeds of *Vicia faba* were used in this experiment. The seeds were soaked for 24 hr in distilled water and germinated up to 2 cm-length primary roots in a Petri dish at temperature 23℃ in the dim light before starting the experiment. On the basis of the preliminary tests, TCB (mol wt, 181.45) which had been dissolved in acetone were added to soil in Petri pots (od=9.5cm, h=10.5cm) to achieve final concentrations: 0, 50, 100, 200, 300 μg g^{-1} with three replicas. The surface soil (0-20cm) with organic matter 2.21% and total nitrogen 0.12% was selected from soybean field at the Shenyang Ecological Experimental Station of

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Chinese Academy of Sciences. The TCB used in this experiment was of analytical grade from Fluka Chemie Company, Germany with purity more than 99%. The final concentration of acetone in each treatment and in the control was 0.9%. Seedlings with 2cm-length primary roots were treated with each TCB concentration and grown at 23℃ and sampled once each 24 hr, until 96 hr. In each treatment, lengths and diameters of total 20 treated primary roots were measured at 24, 48, 72, 96 hr. Seedlings were then transferred to fresh water to recover respectively for 17 hr in chromosomal aberration experiment and for 20 hr in micronuclei observations. Samples of root tips were cut, then fixed in a mixture of absolute ethanol and glacial acetic acid (3:1) for 24 hr, hydrolysed in 5 mol L^{-1} hydrochloric acid for 23 min at 28℃, Feulgen-stained, squashed in 45% acetic acid (Li et al. 1996). The percentage of chromosomal aberration cells at metaphase and anaphase was scored from five root tips in each treatment (de Kergommesaux 1983). At least 5000 cells were examined for each TCB concentratoion and then the mitotic index was scored. Root length, diameters, and frequencies of aberrant cells and micronucleate cells at different doses were compared statistically with controls at the various durations.

RESULTS AND DISCUSSION

TCB had an inhibitory effect on root growth of *V. faba* seedlings at all concentrations (50-300 μg g^{-1}) used in the experiment (Table 1). Radicle lengths were decreased with the increase of TCB concentration and with the duration of the treatment, indicating a dose-dependent response. It was noticed that the roots stopped growing completely after 48 hr at 300 μg g^{-1} TCB treatment.

Table 1. Effects of TCB stress on root length and diameters of *V. faba* seedlings with time.

TCB concentration (μg g^{-1})	Root diameters* (mm)	Root length (cm)			
		24 hr	48 hr	72 hr	96 hr
0	1.3	3.6	5.2	6.6	8.2
50	1.5	3.4	4.6	5.9	7.0[a]
100	2.4[a]	2.9	3.6[a]	4.2[a]	4.6[b]
200	3.2[b]	2.6[a]	3.0[a]	3.4[a]	3.7[b]
300	3.6[b]	2.4[a]	2.6[b]	2.6[b]	2.5[b]

[a]$P<0.05$; [b]$P<0.01$.The same below. * Measured after 72 hr of treatment.

Effects of TCB on the morphology of roots also varied with TCB concentrations. After 24 hr of treatment, the morphology of the root meristem was normal at 50-100 μg g^{-1} TCB, but was starting to show a slightly twisted appearance at

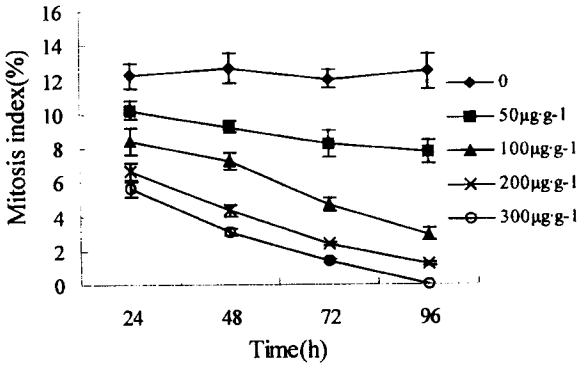

Figure 1. Effects of TCB stress on mitosis index in root-tip cells of V. faba seedlings.

200-300 μg g^{-1} TCB. After 72 hr of treatment, there were significant differences between control and treatment groups of 100-300 μg g^{-1} TCB in the diameters of root tips ($P<0.05$ and $P<0.01$) (Table 1), which could be a compensatory response of root length growth of seedlings inhibited by TCB stress.

The mitotic index reflects the frequency of cell division and is regarded as an important parameter when determining the rate of root growth in plants. The data in Figure 1 showed that with the duration of the treatment, the mitotic index decreased progressively. There was a highly significant difference between the control and 200-300 μg g^{-1} TCB for 48-96 hr ($P<0.05$ or $P<0.01$) or 100 μg g^{-1} TCB for 72-96 hr ($P<0.05$). After 24 hr of TCB treatment, cell division was significantly inhibited and the mitotic index was reduced to 0 at 300 μg g^{-1} TCB for 96 hr. This fits well with the above mentioned effects of TCB stress on root growth, suggesting that the inhibition of root growth resulted from inhibition of the cell division.

The nuclei rupture in some interphase cells of *V. faba* seedling root-tips was found at 300 μg g^{-1} TCB for 72 hr (Figure 2-5). Formation of micronuclei was more common under TCB stress. The results indicated that different TCB treatment induced varied micronuclei effects, frequency of micronuclei was increased with the increase of TCB concentration and treatment duration, and was 0.3~8.2‰ (Table 2). A positive correlation ($r=0.78$-0.93) was obtained between frequency of micronuclei and TCB concentration, but frequency of micronuclei decreased under 300 μg g^{-1} TCB for 96 hr, which could be related to the delayed cell division induced by the higher TCB concentration.

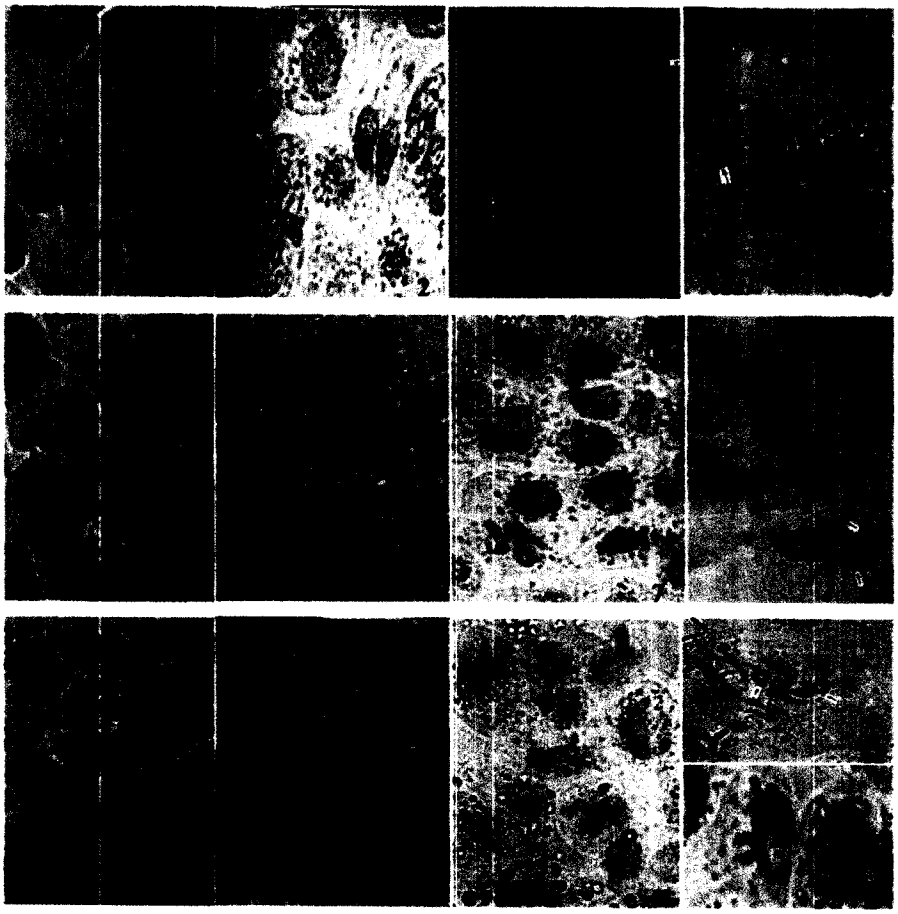

Figure 2. Toxic effects of TCB stress on chromosome of root-tip cells in *V. faba* seedlings (15×100) . (1) Asymmetry array of chromosome at metaphase and c-mitosis (50μg g^{-1}TCB, 48 hr); (2) Stickiness, ring and asymmetry array of chromosome at metaphase (200μg g^{-1}TCB, 24 hr); (3) Chromosome bridge and c-mitosis (50μg g^{-1}TCB, 24 hr); (4) Rings and stickiness of chromosome at metaphase (300μg g^{-1}TCB, 24 hr); (5) Three split nuclei, Chromosome bridge and stickiness at anaphase (300 μg g^{-1}TCB, 72 hr); (6-7) Different kinds of stickiness of chromosome (200-300μg g^{-1}TCB, 48-72 hr); (8-12) Different kinds of ring, break and stickiness of chromosome (200-300μg g^{-1}TCB, 48-72 hr); (13) Micronucleus and stickiness during mitosis (300μg g^{-1}TCB, 48 hr).

Chromosome aberrations have been used as a index of possible genetic damage by environmental toxicants in plants for many years and can provide both qualitative and quantitative data on contamination effects. It was noted that the types of numerical and structural chromosome aberrations during mitotic cell cycles produced by TCB included: c-metaphases; asymmetry array; break; bridge; ring; and sticky chromosomes, depending on the dose and treated time (Figure 2).

Table 2. Effects of TCB stress on root-tip micronucleus frequency of *V. faba* seedlings (‰).

TCB concentration ($\mu g\ g^{-1}$)	Treatment time (hr)			
	24	48	72	96
0	0.36	0.32	0.37	0.34
50	0.33	0.53	0.75	0.71
100	0.32	0.92	1.62[a]	2.82[a]
200	0.59	3.21[b]	4.33[b]	5.98[b]
300	1.92[a]	7.51[b]	8.21[b]	5.05[b]

The data on the frequencies of a different spectrum of chromosome aberrations in root tip cells induced by different concentrations of TCB were presented in Table 3. Lower TCB concentrations (50-100 $\mu g \cdot g^{-1}$) mainly caused c-metaphases, chromosomal asymmetry array and chromosomal bridge, whose percentage in root tips for 24-48 hr of treatment was up to 2.1-11.0%, and also existed in a few cells at higher TCB concentrations (Figure 2-2,5,10). In some of the cells, stickiness and breakage chromosomes were found at metaphase and anaphase (Fig.2-6-13), which indicates a cytologically severely toxic effect, and probably led to the death of the cells.

The percentage of chromosomal stickiness, chromosomal stickiness + chromosomal break, chromosomal stickiness + chromosomal ring, chromosomal stickiness + chromosomal asymmetry in root tips reached 40.1-88.9%, and 19.0-29.6% for different kinds of chromosomal break at 200 and 300 $\mu g\ g^{-1}$ TCB for 24-96 hr.

It is found that with the increase of TCB concentrations, there was a significant increase in the frequencies of total chromosome aberrations (Table 3), and a significant difference ($P<0.01$) was obtained with TCB concentrations above 100 $\mu g\ g^{-1}$. There were no differences between 50 $\mu g\ g^{-1}$ TCB and control ($P>0.05$) except after 72hr of treatment. Table 3 showed that the increase of the frequencies of chromosome stickiness was also dependent on the duration of treatment, and the increase was significant after treatment of 200 and 300 $\mu g\ g^{-1}$ TCB for 24-96 hr and of 100 $\mu g\ g^{-1}$ TCB for 96 hr.

According to the report by Hartwell (1989) whether dividing cells come into the next cell phase depends on the completion of the former cell phase. This dependent regulation mechanism is called checkpoints which are regulatory sites in which many genes and their primary transcript check cell's state including: cell's volume; DNA replication; spindle's assembly; and DNA separation

Table 3. Effects of TCB stress on frequency of chromosomal aberrations of root-tip in *V. faba* seedlings at different time.

Treatment time (hr)	TCB concentration ($\mu g\, g^{-1}$)	Dividing cells.	Anomalous dividing cell (%)								Total (%)
			C	Be	S	B	S+B	B+A, B+R	S+Be, S+A, S +R	A, A+R	
24	0	400	0.4	0.2	0	0	0	0	0	0	0.6
	50	400	1.1	0.3	0	0	0	0	0	0.7	2.1
	100	365	2.4	2.9	3.0	0.4	0	0	0	5.0	13.7[b]
	200	211	1.9	2.8	19.2	2.9	11.2	4.9	9.7	2.5	55.1[b]
	300	97	2.5	0	45.1	3.9	17.1	4.2	16.6	2.8	92.2[b]
48	0	400	0.8	0.1	0	0	0	0	0	0	0.9
	50	390	1.2	0.7	1.0	0	0	0	0	0.6	3.5
	100	370	3.5	0.8	7.6	0.4	0	0	7.5	6.7	26.5[b]
	200	215	4.1	1.4	24.5	2.9	11.8	4.9	14.3	5.0	68.9[b]
	300	83	3.4	1.4	47.0	3.7	17.3	4.5	17.7	3.5	98.5[b]
72	0	400	0.7	0.1	0	0	0	0	0	0	0.8
	50	400	2.8	1.6	2.0	0	0	0	0	0.5	6.9[a]
	100	320	3.3	1.6	9.9	2.8	5.2	2.6	8.1	6.5	40.0[b]
	200	145	2.2	1.5	48.1	3.2	13.3	5.9	7.5	4.0	85.7[b]
	300	69	0	0	58.3	5.5	16.8	6.0	7.2	4.1	97.9[b]
96	0	400	0.7	0.1	0	0	0	0	0	0	0.8
	50	400	1.7	1.1	1.8	0	0	0	0	0.6	5.2
	100	310	3.8	2.7	24.9	3.1	7.4	5.8	10.4	5.1	63.2[b]
	200	125	3.7	2.2	52.0	3.0	12.4	6.2	10.4	0	89.9[b]
	300	27	0	0	55.6	3.7	18.5	7.4	14.8	0	100.0[b]

C, c-mitosis; Be, bridge; S, sticky chromosome; B, break; S+B, sticky + break; A, asymmetry; R, rings.

(Hartwell 1989; Paulovich 1995). If regulation protein checks out the lighter DNA damage in cells, cell cycles will be delayed temporarily to ensure enough time for cells to repair damaged DNA, thus avoiding gene mutation from damaging base or chromosomal aberration from damaging DNA replication. If severe damaged DNA can not be repaired, even cells have completed DNA synthesis, once checkpoints check out chromosomal aberration or abnormality of DNA replication, cells could never enter the mitosis phase of cell cycle (Paulovich 1995). The results in this investigation showed that TCB stress induced chromosome and DNA strand breakage which interferes with cell cycle, thus the number of the dividing cells declined and mitosis index was reduced. The degree of inhibition was increased with increasing TCB concentration and treatment duration, which is identical to the inhibitory effects of ortho- dichlorobenzene on mitotic index of other plants (Brusick 1986).

Some studies showed that TCB is an inhibitor of Ca^{2+}-channel in cell membrane systems and prevents tubulin's aggregation and microtubule's assembly by reducing Ca^{2+} concentration in cytoplasm, thus form c-mitosis due to stopping chromosome development and retaining at the original mitotic phase, or polyploidy cells from chromosome's inseparation (Xia 2001). In this experiment, 50-100μg g^{-1} TCB inhibited mitosis index of root-tip cells in *V. faba* seedlings and significantly increased proportion of c-mitosis (Fig.2-1, 3), showing that lower TCB concentration damaged spindle's formation and function of root-tip cells in *V. faba* seedlings, and induced the change of numeral chromosomes.

It was reported that chromosomes were congressed in equatorial plane by centromere orientation at initial metaphase of mitosis (Rieger et al. 1976). In this experiment chromosomal asymmetry was observed (Figure 2-1, 2), indicating that TCB could make centromere single-oriented distribution by interfering with interaction of sister centromere, spindle and spindle fiber, or make chromosomes at metaphase line unevenly by interfering with re-orientation of chromosome.

TCB was a strong mutagenic pollutants to be oxidized to TCB-4,5-epoxy compound and TCB-5,6-epoxy compound by cytochrome P-450 enzymes (Zhou 1986). The above intermediate compounds with strong chemical activity are easy to combine with $-NH_2$ in guanine producing TCB-DNA adducts, or cause DNA strand breakage directly by acting on DNA template structure, or make chromosomal fiber improperly fold up to single chromotid by affecting –SH and GSH contents in histone molecules and make chromosome become attached to each other by subchromatid bridges (Rieger et al. 1976; Xia 2001; Xiong 2000). In this experiment, TCB stress induced partial stickiness of chromosome in root-tip cells of *V. faba* seedlings (Figure 2-4); while severe TCB stress caused chromosomal stick, thus formed irregular mass shape with single chromosomal

shape vagueness, or induce chromosomal break and ring at the same time of chromosomal stickiness (Figure 2-6, 10-13). Chromosomal stickiness and break has a irreversiblely heavy toxic effect, and could induce further programmed cell death (Xiong 2000).

The highly hydrophobic pollutants easily reach the active cells of the root meristem in plants once they get in touch with the plants. Afterwards, the time producing genotoxicity was mainly during the period of DNA and chromosomal replication in interphase of mitotic cell division (Duan et al. 1995). The visible marker of genetic damage in cytology was detected during the stage of cell division. In general, without activities of mitotic cell division, toxic pollutants have no chance to act upon DNA and express their genotoxicity, thus they can not be detected through cytological genetic methods like chromosomal aberrations and micronuclei frequency (Duan et al. 1995). The results in this experiment showed that in spite of its highly mutagenic characteristics, 300μg g^{-1} TCB for 96 hr induced a lower frequency of micronuclei in root-tip cells of *V. faba* because of lower mitotic index, suggesting that it is necessary to refer to mitotic index under condition of higher concentration of pollutant and longer duration in the meantime, when using frequency of micronuclei as monitoring index under toxicant condition. Among all kinds of chromosomal aberrations in root-tip cells of *V faba* seedlings, the percentage of chromosomal stickiness reached 40.1-88.9%, and 19.0-29.6% for chromosomal break as well as 0.3-8.2% for micronuclei, showing that frequency of micronuclei in *V. faba* root-tip cells can not be substitute for the analysis of chromosomal aberrations that could be used as a sensitive biomarker of monitoring soil contaminated with TCB. As for the effects of TCB on the nuclei, except micronuclei, split nuclei have not been reported in earlier publications. The present experiment showed that *V. faba* seedling was sensitive to the toxic effects of TCB .

TCB stress possesses strong mutagenic property, similar to that in human cells (Xia 2001), indicating that the assessment of TCB genotoxicity by root-tip cytology has good reliability. TCB could induce the percentage of chromosomal breakage less than that of chromosomal stickiness in *V. faba* seedlings, which differs from the result that ortho-dichlorobenzene mainly caused chromosomal breakage in human cells (Zapata et al. 1982). The above results could be involved in reaction characteristics and sensitivity of animal and plant cells to TCB and ortho-dichlorobenzene, and needs to be further investigated.

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