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BRIEF COMMUNICATION

Trichloroacetic acid of different origin in Norway spruce needles and chloroplasts

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Abstract

Trichloroacetic acid (TCA), a secondary atmospheric pollutant, is also formed in forest soil and thus ranked among natural organohalogens. The observed biooxidation of atmospheric tetrachloroethene (PER) to TCA in chloroplasts has led to the investigation of the mode of action of TCA in spruce needles, since TCA is also accumulated in the needles after its rapid uptake from soil by roots. Being phytotoxic, TCA considerably influences conifers by affecting their photosynthetic apparatus. We examined the transport of TCA from soil into chloroplasts in order to compare the effects of TCA on conifers from both sources, *i.e.* endogenously produced within chloroplasts or taken up by roots. The influence of TCA formed in chloroplasts was found to be much more adverse than that of "soil" TCA.

Additional key words: ^{14}C-14 labelling, subcellular level, tetrachloroethene, biooxidation, needle injury.

While extensive research has been focused on the origin, transport, and fate of trichloroacetic acid (TCA) in forest ecosystems, its mode of action remained unknown (Frank *et al.* 1994, Sutinen *et al.* 1995, Forczek *et al.* 2001). TCA may be formed by photooxidation of C_2-chlorocarbons as a secondary atmospheric pollutant (Franklin 1994), by biooxidation of C_2-chlorocarbons within the plant cells (Weissflog *et al*. 2007), or in the soil by enzymatic chlorination of soil organic matter (Matucha *et al*. 2007). Atmospheric precursors of TCA are tetrachloroethene (PER) and 1,1,1-trichloroethane. While emissions of the latter precursor have been diminished during the last decade, PER is still emitted in extensive amounts (McCulloch 2002). In addition to the anthropogenic PER, its natural sources also contribute to elevated concentrations in the atmosphere. Here biomass fires (Weissflog *et al*. 2004), marine algae (Abrahamsson *et al*. 1995), and microbial formation in salt lakes are to be considered (Weissflog *et al*. 2005).

Atmospheric TCA is deposited by precipitation directly into the soil, or by dry deposition to the canopy, from where it is mainly washed down into the soil and contributes to the soil TCA pool. TCA, which is translocated into plant leaves is slowly eliminated either by biodegradation in the phyllosphere, re-transport *via* the phloem (Forczek *et al.* 2004), or by thermal decarboxylation (Matucha *et al.* 2006). Polarity and dissociation of TCA, however, do not allow its passive transport to all cell compartments. If we assume only passive transport, TCA is confined in dissociated form to

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Abbreviations: DCA - dichloroacetic acid; GSH - glutathione; PER - tetrachloroethene; ROS - reactive oxygen species; TCA - trichloroacetic acid.
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the polar cell compartments, probably mainly the vacuole. A different situation occurs when TCA is formed from its precursors inside the plant chloroplasts (Weissflog *et al.* 2007). C_2-chlorocarbons are readily absorbed in the cuticle and any membranes of cells containing lipids and wax substances, from where they can be distributed to mesophyll cells or be desorbed again (Frank and Frank 1986, Figge 1990). Lange *et al.* (2004) found that the phytotoxicity of similar TCA concentrations in pine needles depended on the assimilation pathways of TCA in the plant. TCA translocated into the spruce needles *via* the soil/root pathway displays milder phytotoxic effects than comparable amounts of TCA formed in spruce needles after atmospheric PER exposure. The above authors suggest that TCA transported from the soil accumulated and degraded in vacuoles or in the apoplast, whereas TCA formed directly in the chloroplasts from PER affected enzymes of the photosynthetic apparatus and thus exhibits much stronger phytotoxicity. Weissflog *et al.* (2007) showed that no dichloroacetic acid was formed in the course of PER metabolism. This permitted the conclusion that PER degradation in pine trees under drought stress proceeded only *via* oxidative transformation and not *via* GSH induced conjugative biotransformation. Biooxidation of PER in spruce needles has been indicated recently (Weissflog *et al.* 2007). The aim of this work was to clarify if there is a difference in subcellular localization between TCA taken up by the root-xylem-needles pathway and TCA formed directly in needle cells by oxidation in chloroplasts. Isolation of chloroplasts after application of radioactively labelled [1,2-^{14}C]PER and [1,2-^{14}C]TCA could provide a direct proof of the site of TCA accumulation in both cases.

Four-year-old Norway spruce [*Picea abies* (L.) Karst.] saplings were obtained by vegetative propagation of spruce from Ore Mountains, Czech Republic. [1,2-^{14}C]TCA of radiochemical purity >99 % and specific activity 3.7 GBq mmol^{-1} prepared according to Bubner *et al.* (2001) and [1,2-^{14}C]tetrachloroethene of radiochemical purity >98 % and specific activity of 240 MBq mmol^{-1} (*ARC*, St. Louis, MO, USA) were used for radiotracer study. Exposure of spruce saplings to [1,2-^{14}C]PER was conducted in an air-tight 26-dm^3 glass chamber. Radioactive PER was applied in an open Petri dish placed under the branches of the tree mixed in 1 cm^3 *n*-hexadecane for even and slow evaporation into the atmosphere. The photoperiod was 12 h, irradiance of 150 μmol m^{-2} s^{-1} provided by two 75 W reflector lamps (*Paulmann*, Springe, Germany) placed outside the glass chamber, and temperature 25 °C. The saplings were exposed to 2.83 MBq [1,2-^{14}C]PER for 13 d and to 3.40 MBq [1,2-^{14}C]PER for 16 d. To simulate uptake of TCA from the soil (Forczek *et al.* 2004), detached shoots (fresh mass 2 - 5 g) of 4-year-old cuttings of Norway spruce (of the same provenance as in PER exposure experiments) were placed into [1,2-^{14}C]TCA-solution (510 and 798 kBq) in 500 cm^3 Erlenmeyer flasks for 7 d. The youngest 30 - 40 mm elongated current-year-shoots (C) and one-year-old needles (C+1) were used for TCA determination.

Chloroform, *n*-heptane, KH_2PO_4 (*Merck*, Darmstadt, Germany), 8 cm^3 corning screw cap vials and a water bath were used for determination of [1,2-^{14}C]PER and [1,2-^{14}C]TCA by the decarboxylation method (Matucha *et al.* 2006). The amount of radioactive PER was determined by scintillation counting following extraction of [1,2-^{14}C]PER into 5 % chloroform in *n*-heptane-solution at 60 ºC. In a second step, the amount of [1,2-^{14}C]TCA was determined by scintillation after a 2-h thermal decarboxylation of [1,2-^{14}C]TCA to ^{14}C-chloroform and $^{14}CO_2$ at 100 ºC. A liquid scintillation spectrometer (*Beckman LS 6500*, Fullerton, CA, USA) and *Rotiszint Eco Plus* scintillation cocktail (*Carl Roth*, Karlsruhe, Germany) were used for radioactivity measurements. The mean radioactivity of the measured ^{14}C-chloroform was then multiplied by two as only half of the amount of the uniformly labelled [1,2-^{14}C]TCA (as well as of original [1,2-^{14}C]PER) yields ^{14}C-chloroform by thermal decarboxylation. The total PER and TCA content of current needles (C) and older needles (C+1, C+2) were determined separately.

The isolation of chloroplasts was described recently (Weissflog *et al.* 2007). The preparation medium contained 0.4 M sucrose, 20 % (m/m) PEG-4000, 50 mM HEPES (pH 7.6), 10 mM NaCl, and 5 mM $MgCl_2$. The method used (Martin *et al.* 1978) is based on rapid (5 s) homogenization of finely chopped needles (5 mm pieces; 2.5 g) by *Ultra-Turrax* (*IKA Labortechnik*, Staufen, Germany) in a relatively large volume (40 cm^3) of ice-cold preparation medium. All centrifugation steps were performed at 4 ºC. The homogenate was filtered through a single layer of *Miracloth*® and centrifuged for 3 min at 6 000 *g*. Pelleted intact chloroplasts were re-suspended in 15 cm^3 preparation medium and centrifuged again for 10 min at 3 000 *g*. For the final cleaning step, the pellet was re-suspended again in 15 cm^3 preparation medium and centrifuged for 2 min at 150 *g* to pellet the debris. The supernatant was centrifuged for 10 min at 3 000 *g* and the pellet consisting of clean chloroplasts re-suspended and kept in a storage medium consisting of 1.2 M sucrose and 50 mM HEPES (pH 6.9). All measurements were made in triplicate, the pooled standard deviations are given in the tables.

Investigations of effects of phytotoxic TCA on conifers never provided linear dose-response relationship between the TCA content and the injury to the coniferous forest (Frank *et al.* 1994, Plümacher *et al.* 1994, Norokorpi and Frank 1995). This might be caused either by the polyfactorial character of forest damage and/or by TCA acting differently on whole plant and subcellular level. TCA is absorbed by roots, bark, and stomata (especially from fog droplets), the uptake through intact cuticle wax layer is improbable. The other possibility, indicated already by Figge (1990), who found that CO_2 is released from spruce needles after application of PER, is TCA formation by the biooxidation of the PER

Table 1. Amount of 1,2-^{14}C]TCA in needles and in isolated chloroplasts (± pooled SD) after TCA uptake and translocation by the transpiration stream (simulating uptake from soil).

		Experiment 1 [Bq g^{-1}(needles f.m.)]	TCA [%]	Experiment 2 [Bq g^{-1}(needles f.m.)]	TCA [%]
Needles	C	42055.1 ± 3715.7	100 ± 8.8	100122.9 ± 3969.1	100 ± 4.0
	C+1	22919.4 ± 623.4	100 ± 2.7	80247.5 ± 9559.8	100 ± 11.9
Chloroplasts	C	6.8 ± 3.1	0.016 ± 0.007	99.0 ± 53.0	0.099 ± 0.053
	C+1	4.4 ± 1.8	0.035 ± 0.008	95.3 ± 12.5	0.119 ± 0.016

Table 2. Amount of [1,2-^{14}C]TCA and [1,2-^{14}C]PER in needles and chloroplasts of C and C+1 needle year classes after exposure of spruce saplings to vapours of [1,2-^{14}C]PER (values ± pooled SD).

Needles	Experiment	Needles [Bq g^{-1}(needles f.m.)] TCA	PER	Chloroplasts [Bq g^{-1}(needles f.m.)] TCA	PER
C	3	1805.0 ± 274.0	12511 ± 2028	11.6 ± 1.8	17.8 ± 0.9
	4	666.0 ± 91.6	10726 ± 1225	53.8 ± 2.9	222.9 ± 4.1
C+1	3	108.5 ± 49.6	197.5 ± 2.0	1.8 ± 0.1	0.7 ± 0.1
	4	134.9 ± 9.4	750.7 ± 47.3	13.0 ± 1.6	17.0 ± 2.5

penetrated into chloroplasts (Weissflog *et al.* 2007). The higher impact of PER (and TCA formed from it) on photosynthesis and the vitality of conifers (Frank and Frank 1986) as compared to TCA taken up by roots is thus understandable. It is supposed that after uptake by roots, TCA is transported in the xylem, accumulated in needle parenchyma cells, and then slowly decomposed by biodegradation (Forczek *et al.* 2004) and thermal decarboxylation (Matucha *et al.* 2006).

The penetration of PER into spruce needles and their chloroplasts together with its bio-oxidation to TCA was therefore compared with the transport of the TCA from the soil. A spruce sapling was exposed to [1,2-^{14}C]PER for 9 d in a closed chamber and the chloroplasts were then isolated from a suspension of homogenized needle cells. The isolation of chloroplasts resulted in low yield, most of the radioactivity loss occurring during the first step, *i.e.* after disintegration of the needles, while during further isolation steps no considerable radioactivity was lost (Weissflog *et al.* 2007).

In comparison to PER exposure, TCA transported by roots and xylem and finally accumulated in needles (Table 1), was not able penetrate into chloroplasts as easily as PER (Table 2). Only traces of TCA were found in chloroplasts after a week of exposure (Table 1). We thus may assume that polar and dissociated TCA cannot readily enter chloroplasts through the cuticle, cell membranes, and cytoplasm. A very low amount of radioactivity was present in the isolated chloroplasts of both needle ages (in both experiments, see Table 1). Hence TCA taken up by roots behaved differently from TCA formed by biodegradation of PER. This can also explain the direct visible effect of these compounds on the plants. PER (and also the TCA formed) causes chlorotic and necrotic spots on the needles, while TCA alone, when applied directly onto leaves or taken up by roots, causes visible symptoms only at higher concentrations (Sutinen *et al.* 1995).

As PER is soluble in apolar substances, it might be taken up by the cuticle and an equilibrium is established between the air and leaves (Figge 1990). This balance, however, can be shifted to the uptake process, when PER is transformed to TCA, steadily decreasing the concentration of PER present in needles. The amount of ^{14}C-PER taken up was dependent on its concentration in the atmosphere and the total biomass of the needles in the tree crown. Almost all of the applied ^{14}C-PER was taken up into the needles after the exposures. Control samples showed only low residual radioactivity in the *n*-hexadecane (0.07 %) and in the atmosphere (5.2 % absorbed on *TENAX*). The amount of TCA formed in isolated chloroplasts is *ca*. three times higher than in whole needles. This fact points to the PER → TCA transformation taking place in the chloroplasts. Older needles (C+1) showed lower amounts of PER taken up but the conversion (43 - 72 %) was higher than in current needles (Table 2). This can be explained by decreased activity or amount of detoxification enzymes, or by the fact that TCA is not transported out of chloroplasts at such a rate as in younger cells.

Our experiments showed that uptake of phytotoxic TCA from the did not lead to noticeable translocation into chloroplasts, however, may conceivably exert an effect on needle cells by changing cell pH as it is mineralized to HCl and CO_2. Decrease of pH can lead to structural and functional changes, *e.g.* in membranes and enzymes,

which finally adversely affect photosynthesis. TCA formed directly in the chloroplasts leads to damage of the photosynthetic apparatus because TCA prompts protein protonation. Obviously, PER might be thus more dangerous to spruce than TCA – its atmospheric degradation product. It is striking to see that subcellular localization makes a large difference in the mode of action of the pollutants.

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