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Oxidative biodegradation of tetrachloroethene in needles of Norway spruce (*Picea abies* L.)

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Abstract

Through employing [^{14}C]-PER exposure experiments it was shown for the first time that PER taken up by drought-stressed spruce needles via the *air/needle* pathway is preferably degraded to trichloroacetic acid (TCA) in the chloroplasts. TCA formed by oxidative biotransformation is mineralised to CO_2 and HCl via various degradation routes.

HCl contributes to increased proton concentration in the chloroplast, inducing a pH shift leading to a pathophysiological effect on H^+ transport from the thylakoid interior into the stroma. As a result of their high degree of dissociation and related protonation, the PER metabolites, TCA and HCl, cause a change in protein structures. In addition to this, the TCA anions created in the process may lead to destabilisation of the thylakoid membrane potential. The damage to the chloroplasts inflicted by protons and trichloroacetate ions subsequently leads to an impairment of photosynthesis, most particularly to uncoupling of photosynthetic electron transport. Since progressive aridity as consequence of the climate change observed throughout the world is predicted, a regionally variable marked enhancement of the phytotoxic risk caused by PER emission is anticipated.

Keywords: Biodegradation; Chloroplasts; Drought stress; Norway spruce; Tetrachloroethene; Trichloroacetic acid

1. Introduction

Tetrachloroethene (PER) is of considerable importance as a degreasing and cleaning agent in the metal-processing and textile industries because of its physicochemical properties. Approximately 300 kt were industrially produced worldwide in 1996 (Midgley and McCulloch, 1999), when industrial production ceased due to concerns about the involvement of chlorohydrocarbons in climate change. Still, extensive amounts of PER are emitted in the processes of bleaching raw cellulose (Juuti et al., 1995, 1993), incinerating chlorinated plastics (Weissflog et al., 2004) and the combustion of coal containing chloride in power stations (Garcia et al., 1992). Among natural sources, microbial production in forest soil (Hoekstra, 1999; Hoekstra et al., 1998) and formation in biomass fires (Weissflog et al., 2004; Rudolph et al., 2000) are the predominant producers of PER. The microbial formation of PER and other C_1/C_2 chlorohydrocarbons in salt lakes has been shown by Weissflog et al. (2005a).

Forests are a strong sink for volatile chlorohydrocarbons. As a result of the large leaf area index and the cuticular properties of many plants, high scavenging rates for atmospheric organic xenobiotics (Gaggi et al., 1985; Plümacher and Schröder, 1994; Plümacher et al., 1994) may be observed.

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Table 1
Oxidation potential of several oxidants in water (CRC Handbook, 1985)

Oxidant	Oxidation potential (eV)
Hydroxyl radical, •OH	2.80
Singlet oxygen, O(1D)	2.42
Ozone, O_3	2.07
Hydrogen peroxide, H_2O_2	1.77
Superoxide anionic radical, $•O^{2-}$	1.70
Oxygen, O_2	1.23

The atmospheric decomposition of PER by oxidative radical reactions, mainly to phytotoxic trichloroacetic acid (TCA), has been studied fairly extensively (Folberth et al., 2003; Sidebottom and Franklin, 1996; Franklin, 1994), and about a possible correlation between PER and TCA content in natural forests has also been reported (Plümacher and Schröder, 1994).

Whereas PER metabolism in humans and in mammals (De Raat, 2003) has been described in detail, comparable and conclusive studies on the uptake, distribution and metabolism of PER in terrestrial plants have so far not been done. PER can be taken up by terrestrial plants via both the *soil/root* pathway and the *air/leaf* pathway. The uptake of PER via the *soil/root* pathway in willows, poplars and cotton plants has been examined within the scope of phytoremediation experiments (e.g., Nzengung and Jeffers, 2001; Nzengung et al., 2000; Gordon et al., 1998). Using well-watered plants, these authors found that the conjugative biodegradation of PER to dichloroacetic acid (DCA) by reduced glutathione (GSH) taking place in the plant is the preferable pathway. DCA has a lower phytotoxicity than TCA.

Franzaring et al. (2000), Kotte (2004), Lange (2005) and Lange et al. (2004) studied the chronic uptake of PER via the *air/leaf* pathway in a variety of different plant species and detected phytotoxic metabolites such as TCA in the plant. Comprehension of PER metabolism by plants under environmentally relevant conditions (incl. drought stress) was contributed to by Lange (2005), Kotte (2004), Lange et al. (2004), Strauss et al. (2004) and Weissflog et al. (2004, 2003).

With regard to the uptake of PER via the *air/leaf* pathway, two mechanisms can be distinguished. In the first case, PER is taken up by diffusion processes via the stomata directly into the mesophyll cells (Kotte, 2004; cf. Cornejo et al., 1999). In the other case, PER is accumulated from the surrounding atmosphere and enriched around 2200 times in the lipid/wax components of spruce needles (Frank and Frank, 1986). The adsorption/desorption equilibrium of this process is reached after an hour. (Figge, 1990). Considerable efforts have been made to determine deposition rates (Schröder and Weiss, 1991; Schröder, 1998). Evidence was obtained for the accumulation in the cuticle and uptake of volatile chlorohydrocarbons into the needles under ambient conditions. For tetrachloromethane, tri- and tetrachloroethene, deposition velocities of 0.1 mm s^{-1} were determined (Figge, 1990). PER accumulated in the epicuticular wax layers may subsequently either diffuse directly through the wax layers into the interior of the needles or may return to the surrounding atmosphere by desorption when low atmospheric mixing ratios prevail, wherefrom a direct stomatal *re*-diffusion may also occur. Independent of which of the two uptake mechanisms dominates, PER diffuses into all needle cells, accumulating predominantly in lipid-containing cell compartments (chloroplasts among others).

Strong light and drought stress lead to *photo-inhibition* and *photo-oxidative stress* in plants. During photo-inhibition, the photosynthetic apparatus is over-saturated with light energy. The exited chlorophyll molecules transfer energy to triplet oxygen ($^3O_2$), leading to the formation of highly reactive singlet oxygen ($^1O_2$). In addition, photo-inhibition leads to an oversupply of electrons in the photosynthetic apparatus, reacting with the $^3O_2$ oxygen present in the chloroplasts, leading to the generation of H_2O_2 and two oxygen radicals, namely, the superoxide anionic radical ($•O_2^-$) and the hydroxyl radical (•OH). $^1O_2$, $•O_2^-$, H_2O_2 and •OH are reactive oxygen species (ROS) toxic to plant cells (Apel and Hirt, 2004). Table 1 shows the oxidation potentials of different ROS in water (CRC Handbook, 1985). Plants normally possess appropriate protective mechanisms for the detoxification of ROS. These are enhanced synthesis of superoxide dismutase (SOD), enzymes of the ascorbate redox chain and other radical scavengers and increased formation of the GSH substrate that lead to the development of a higher detoxification potential in plant cells. GSH is the major low-molecular-weight thiol compound to be found in most plants, acting as a protein disulphide reductant, which detoxifies xenobiotics by the process of conjugation (Arora et al., 2002). Under strong light and drought conditions, GSH is often not available in adequate concentrations for the prevention of photo-oxidative, toxic effects on plant cells (Tausz et al., 2004). Furthermore, drought stress influences the activity of radical detoxification enzyme systems by an increase in lipid peroxidation and the associated membrane damage (Jiang and Huang, 2001; Dat et al., 1998; Jagtap and Bhargava, 1995; Zhang and Kirkham, 1994; Chowdhury and Choudhuri, 1985). Under such conditions, the reduced detoxification of ROS as well as the conjugative detoxification of PER by GSH may no longer function either, or at best, with decreased efficiency.

ROS formed in plant cells–augmented by the disturbance of appropriate detoxification mechanisms–are also able to react with xenobiotics (e.g., PER) taken up by the plant via the pathway described above. Table 2 shows the reaction rate constants for reactions of O_3 and •OH with organic compounds in water (Cater et al., 1990; Dussert, 1997). Particularly conspicuous are the high reaction rates of chlorinated alkenes quoted in Table 2, such as PER with •OH.

Table 2
Reaction rate constants for O_3 and •OH reactions with organic compounds in water (Cater et al., 1990; Dussert, 1997)

Organic compounds	Rate constant (M^{-1} s^{-1})	
	O_3	•OH
Alcohols	10^{-2}–1	10^8–10^9
Aldehydes	10	10^9
Carboxylic acids	10^{-3}–10^{-2}	10^7–10^9
Chlorinated alkenes	10^{-1}–10^3	10^9–10^{11}
Sulfur-containing organics	10–1.6×10^3	10^9–10^{10}

We therefore assumed the point of view that an increased oxidation potential, particularly in the chloroplasts during drought or light stress (Schopfer and Brennicke, 1999), lead to enhanced oxidative degradation of PER to TCA (Weissflog et al., 2001). The latter degradation pathway resembles the well-known oxidative degradation of PER in the atmosphere. In this paper, we describe experiments exposing spruce saplings to [^{14}C]-PER in order to shed some light on the assumed drought stress-induced change from a conjugative to an oxidative biodegradation pathway, as well as on the ecological consequences arising from this process.

2. Materials and methods

Four-year-old Norway spruce (*Picea abies* L.) saplings were obtained by vegetative propagation of spruce of known origin (Kálek, Ore Mts., Czech Republic). The exposure of spruce saplings to [^{14}C]-PER was conducted in a 26-l bell shaped glass chamber placed on a glass plate sealed with concentrated H_3PO_4.

Corning screw cap vials and a water bath were used for determination of [^{14}C]-PER and [1,2-^{14}C]-TCA by the decarboxylation method. PER (Lachema, Brno, CZ) was used for preliminary non-radioactive experiments and [1,2-^{14}C]-PER of radiochemical purity 98% and specific activity of 240 MBq mmol^{-1} (ARC St. Louis, MO, USA) was used for radiotracer studies. [1,2-^{14}C]-TCA was synthesised according to the procedure of Bubner et al. (2001). A liquid scintillation (LS) spectrometer (Beckman LS 6500, Fullerton, CA, USA) and Rotiscint eco plus scintillation cocktail (Carl Roth, Karlsruhe, Germany) were used for measurements of radioactivity.

For the determination of [^{14}C]-PER a modified extraction/decarboxylation method was used (Matucha et al., 2006). Here, first [1,2-^{14}C]-PER is extracted with *n*-heptane containing 5% chloroform at 60 °C from a suspension of disintegrated needles or chloroplasts, respectively, in 4 ml of 1 M phosphate buffer (pH 4.6), and counted by LS. The remaining activity, determined then after 2 h thermal decarboxylation at 90 °C and extraction with *n*-heptane containing 5% chloroform by LS counting of an aliquot of the extract, represents a half of decarboxylated [1,2-^{14}C]-TCA. An aliquot of the upper organic phase (1 ml of 3 ml *n*-heptane–chloroform extract) was carefully pipetted (in duplicate) into a scintillation vial, mixed with 5 ml scintillation cocktail and the radioactivity was counted by LS. The mean radioactivity of [^{14}C]-chloroform measured was then multiplied by two (for exact uniform [1,2-^{14}C]-TCA-labeling used). Identification of [1,2-^{14}C]-TCA was conducted by TLC on cellulose F foils (Merck). The chromatogram was developed with the system *n*-butanol–water–ammonia (85:14:1).

3. [^{14}C]-PER exposure

3.1. First experiment

The aim of the first experiment was to qualify and to optimise (i) the isolation procedure of chloroplasts from spruce needles and (ii) the analytical detection of [^{14}C]-TCA in the chloroplasts after exposure of the plants to [^{14}C]-PER. A 4-year-old Norway spruce was exposed to 3.7 MBq radioactively labelled [^{14}C]-PER (i.e., 2554 μg PER). The spruce was properly irrigated before the start of the experiment, except during the last 3 days (drought stress). The sapling was subsequently kept in a 26-l closed glass chamber without further aeration or irrigation for a further 9 days. [^{14}C]-PER was applied in 1 ml *n*-hexadecane in an open Petri dish placed on the top soil under the branches of the tree for even and slow evaporation into the atmosphere. Illumination was provided by two 75-W plant reflector lamps outside the glass chamber for 12 h/day. The spruce was kept in the open air for 1 day after exposure before sampling of C-, C+1- and C+2 needles was started and investigation of ^{14}C-speciation (chemical form of ^{14}C) was conducted.

3.2. Second experiment

A short period after dormancy, another 4-year-old Norway spruce was transferred into the laboratory and acclimatised for 1 month. Here, the tree started to grow new shoots which were 2–5 cm long. The tree was exposed to 2.83 MBq [^{14}C]-PER for 13 days. After exposure, the air of the chamber was absorbed onto TENAX GC 60/80 polymer sorbent. Light, temperature and irrigation conditions were the same as in the first experiment, i.e., inducing drought stress. The remaining radioactivity of [^{14}C]-PER in *n*-hexadecane phase was 2.1 kBq. The residual radioactivity absorbed from the atmosphere was 0.15 MBq.

3.3. Third experiment

The aim of the third experiment was to verify the previous results under similar conditions. A 4-year-old Norway spruce was taken into the laboratory and acclimatised for 1 month. The tree started to grow new shoots which were 4–6 cm long. The tree was exposed to 3.40 MBq [^{14}C]-PER for 16 days. Light, temperature and irrigation conditions were the same as in experiment 1. C+1 needle samples were taken after 9 days.

4. Isolation of chloroplasts and [^{14}C] radioactivity speciation

Radioactivity in the spruce needles was determined by several methods enabling us to specify the chemical form and location of ^{14}C. In order to confirm the working hypothesis, namely, that PER is translocated to the chloroplasts and transformed here to TCA, chloroplasts were isolated.

Chloroplast extraction from spruce needles is difficult because of the high concentration of resins and phenolic compounds present, resulting in a low yield of clean, intact chloroplasts in the final preparation. The preparation medium contained 0.4 M sucrose, 20% (w/w) 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 homogenisation of finely chopped needles (5 mm pieces; 2.5 g) by Ultra-Turrax (5 s) in a relatively large amount (40 ml) of ice-cold preparation

Table 3
Isolation of chloroplasts from current (C) and older (C+1 and C+2) spruce needles after exposure of a spruce sapling to [^{14}C]-PER

Compartment	C needles		C+1 and C+2 needles	
	(Bq/g)	(%)	(Bq/g)	(%)
First supernatant	367.9	71.9	480.8	77.9
Second supernatant	12.7	2.5	19.3	3.1
Third supernatant	3.4	0.7	3.2	0.5
Pellets (chloroplasts)	121.2	23.7	109.2	17.7
[^{14}C]-TCA in chloroplasts	6.5	1.3	4.5	0.7

A spruce sapling was exposed to 3.7 MBq [^{14}C]-PER for 9 days in a 26-l closed chamber, chloroplasts were isolated from suspension of homogenised needle cells. Radioactivity of the fractions shows the proportions of the steps of the isolation procedure (for details, see Materials and methods).

medium. All centrifugation steps required for isolation of the chloroplasts were performed at 4 °C. The homogenate was filtered through a single layer of Miracloth® and centrifuged for 3 min at 6000×*g* (first supernatant in Table 3). The pellet containing intact chloroplasts was re-suspended in 15 ml preparation medium and centrifuged again for 10 min at 3000×*g* (second supernatant in Table 3). For the final cleaning step, the obtained pellet was resuspended in 15 ml preparation medium and centrifuged again for 2 min at 150×*g* to pellet debris only which were then discarded. The third supernatant containing chloroplasts was centrifuged for 10 min at 3000×*g* (radioactivity of the third supernatant in Table 3). The obtained pellet containing clean and intact chloroplasts was resuspended and kept in storage medium. The radioactivity of the chloroplasts pellets (Table 3) was measured and analysed for PER and TCA. The storage medium contained 1.2 M sucrose and 50 mM HEPES (pH 6.9).

[^{14}C]-PER present in supernatants or chloroplasts was quantified by the modified extraction and decarboxylation method described above ([^{14}C]-PER extracted at 60 °C). The formed [^{14}C]-TCA was determined by counting the radioactivity of [^{14}C]-chloroform produced by thermal decarboxylation at 90 °C. The radioactivity of the three supernatants during the extraction procedure and of the purified chloroplasts was also determined by LS counting. The amount of [^{14}C]-chloroform was determined by LS of the *n*-heptane solution of chloroform. Total PER and TCA content of current needles (C) and older needles (C+1, C+2) were determined separately.

5. Results and discussion

The results of the experiments show that [^{14}C]-TCA is formed as key metabolite in the chloroplasts of the needles of spruce trees subjected to drought and light stress and exposed to uniformly labelled [^{14}C]-PER. The results, based on the analysis by the extraction/decarboxylation method described under Materials and methods, were additionally confirmed by radio-TLC of the chloroplast extract. The radioactive spot of its TLC-chromatogram proved to have the same R_f value as that of [1,2-^{14}C]-TCA.

In the first experiment, chloroplasts from current (C) needles contained higher radioactivity as well as a higher percentage of [^{14}C]-TCA than older (C+1) needles (Table 3), indicating the overall higher metabolism of the young needles. The results demonstrate the suitability of the methods used for extraction of chloroplasts and detection of different degradation products of [^{14}C]-PER.

Table 4 shows the absolute distribution of both [^{14}C]-PER and [^{14}C]-TCA in the needles and chloroplasts, as well as their percentage distribution in these compartments after 13 or 16 days exposure to [^{14}C]-PER, respectively, under induced drought stress conditions. A 14% conversion of [^{14}C]-PER to [^{14}C]-TCA is observed in the young needles (C), whereas the older needles (C+1) contain more than 50% of the metabolite.

The results can be understood better by comparison of the results from the second and third experiments (Table 4). The results also show an age-dependent influence on the rates of biodegradation of [^{14}C]-PER to [^{14}C]-TCA by the needle age groups C and C+1. Here, it is perceived that older needles are more decisively driven into the biodegradation alternative by the experimentally induced drought stress, probably accompanied by the more extensive formation of reactive oxygen species (ROS). This may in turn lead to an increased degradation of [^{14}C]-PER to [^{14}C]-TCA in the needles of the age group C+1.

Furthermore, it was indicated as before that no dichloroacetic acid (DCA) is formed in the course of metabolism under the experimental conditions used. This in turn permits the conclusion that the degradation of PER under the influence of drought stress takes place only via oxidative degradation and not via GSH-induced conjugative biodegradation. PER oxidation is most probably mediated by the active oxygen species, H_2O_2 and $^{\bullet}OH$, formed in the thylakoid membrane of chloroplasts inter alia as a result of

Table 4
The radioactivity of [^{14}C]-TCA and [^{14}C]-PER, expressed in Bq g^{-1}, found in chloroplasts and needles of C and C+1 needle year classes after exposure of a spruce sapling to vapors of [^{14}C]-PER (Bq g^{-1})

Age of needles	Needles (total)				Chloroplasts			
	[^{14}C]-PER)/[^{14}C]-TCA				[^{14}C]-PER)/[^{14}C]-TCA			
	Second experiment	(%)	Third experiment	(%)	Second experiment	(%)	Third experiment	(%)
C	12,510.5/1805.0	14.4	10,725.5/666.0	6.2	17.8/11.6	65.0	222.9/53.8	24.1
C+1	197.5/108.5	54.0	750.7/134.9	17.0	1.8/1.7	94.4	17.0/13.0	76.5

The saplings were exposed in the second experiment to 2.83 MBq [^{14}C]-PER for 9 days and in the third experiment to 3.40 MBq [^{14}C]-PER for 16 days in a 26-l closed chamber (for details, see Materials and methods). Percentage formation of TCA by oxidative biodegradation of PER is indicated.

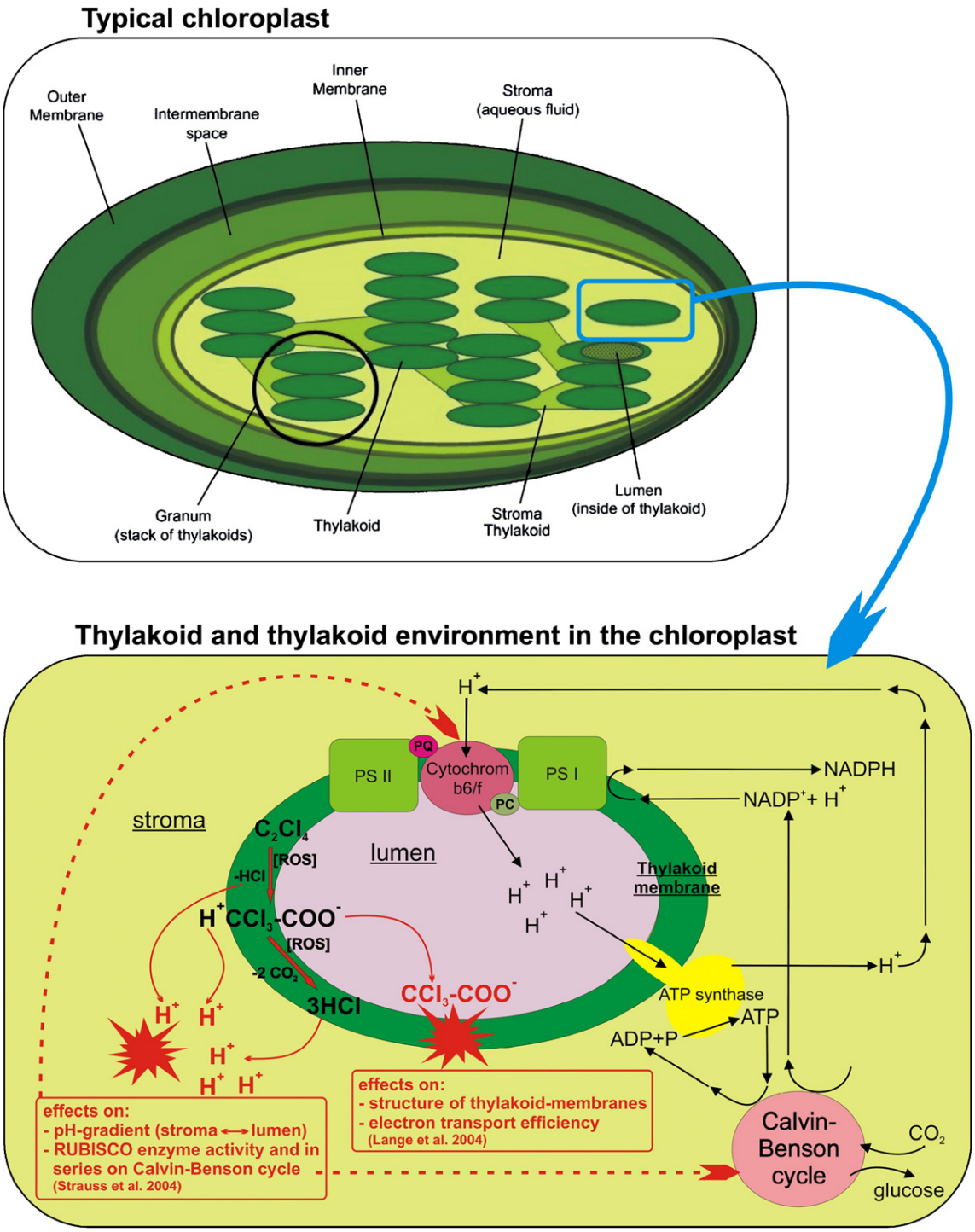

Fig. 1. Schematic presentation of the biodegradation of tetrachloroethene (PER) and implication of its metabolic products in drought-stressed pine needle chloroplasts. Figure changed according to: http://en.wikipedia.Zorg/wiki/Chloroplast, 24.06.2006.

drought stress and react according to the following reaction scheme:

$$C_2Cl_4 + H{-}O{-}O{-}H \rightarrow [\text{tetrachloroxirane}] + H_2O \quad (R.1)$$

$$C_2Cl_4 + {}^{\bullet}O_2H \rightarrow [\text{tetrachloroxirane}] + {}^{\bullet}OH \quad (R.2)$$

The hypothetical intermediate tetrachloroxirane, the formation of which was first suggested by Yllner (1961), deposits in the form of trichloroacetyl chloride ($Cl_3C{-}COCl$) as shown in (R.3), which in turn reacts with H_2O to form TCA and HCl (R.4).

$$[\text{tetrachloroxirane}] \rightarrow CCl_3{-}COCl \quad (R.3)$$

$$CCl_3{-}COCl + H_2O \rightarrow CCl_3{-}COO^- + Cl^- + 2H^+ \quad (R.4)$$

It was further discerned that spruce and pine needles contaminated with TCA exhibit a higher chloroform ($CHCl_3$)

concentration, positively confirming the natural decarboxylation of TCA to $CHCl_3$ in the plant metabolic system (Matucha et al., 2006; Weissflog et al., 2005b). TCA created in this way (in thylakoid membranes of chloroplasts) may be degraded on different pathways to $CHCl_3$ and CO_2 by thermal decarboxylation (Matucha et al., 2006) (R.5), photodecarboxylation (R.6a) (R.6b) (R.6c) and even by enzymatic decarboxylation (E.C. 4.1.1.x) (R.5), although no direct evidence is available for the latter step.

(i) Both thermal and enzymatic decarboxylation:

$$Cl_3C{-}COOH \rightarrow CHCl_3 + CO_2 \quad (R.5)$$

(ii) Photodecarboxylation may be followed by complex formation of the trichloroacetate carboxyl group according to

$$Fe(III)(RCO_2)^{2+} + h\nu \rightarrow Fe(II) + CO_2 + {}^{\bullet}R \quad (R.6a)$$

$${}^{\bullet}OH + Fe(II) \rightarrow Fe(III) + OH^- \quad (R.6b)$$

$$[Fe(III)(Cl_3C{-}COO^-)]^{2+} + h\nu \rightarrow Fe(II) + CO_2 + {}^{\bullet}CCl_3 \quad (R.6c)$$

The unstable intermediate product, trichloromethanol (CCl_3OH), created in the thylakoid membranes of chloroplasts according to reaction (R.7) subsequently decays with the formation of phosgene ($COCl_2$) and HCl. Phosgene in turn hydrolyses to carbon dioxide (CO_2) and HCl rapidly (R.8).

$${}^{\bullet}CCl_3 + {}^{\bullet}OH \rightarrow [CCl_3OH] \rightarrow COCl_2 + H^+ + Cl^- \quad (R.7)$$

$$COCl_2 + H_2O \rightarrow CO_2 + 2H^+ + 2Cl^- \quad (R.8)$$

As a result of the high rate constant of the ${}^{\bullet}OH$ reaction with $CHCl_3$ in water 5×10^6 l mol^{-1} s^{-1}, and the assumed diminished GSH content (induced by the additional drought stress), oxidation of the $CHCl_3$ (formed beforehand (R.5)) to trichloromethyl-radical (${}^{\bullet}CCl_3$) (R9) occurs as well as further reaction of the trichloromethyl radical to phosgene ($COCl_2$), according to (R.7).

$$CHCl_3 + {}^{\bullet}OH \rightarrow {}^{\bullet}CCl_3 + H_2O \quad (R.9)$$

Pohl et al. (1984, 1981) published this reaction pathway in the sequence (R.9) (R.7) (R.8), which also covers the degradation of chloroform in mammals.

Since diffusion or active transport of the formed TCA between chloroplasts (thylakoid interior) and cytoplasm is unlikely, it is assumed that besides production of a CO_2 molecule available for photosynthesis, the mineralisation of PER involves the formation of up to five protons, with immediate toxic effects (see (R.4) (R.7) and (R.8)).

Because of the different chemical nature of the reactions and the different reaction location in the chloroplast (thylakoid membrane and stroma) the formation of the five protons during the degradation of PER ((R.4) (R.7) and (R.8) is not a synchronized process.

The TCA formed from PER in the chloroplasts may either exhibit phytotoxic effects itself, e.g., by coordination with sulfhydryl and amino groups of proteins (e.g., Ashton and Crafts, 1981) or by constraints on the chloroplast function by the protons (H^+) formed by dissociation of TCA and HCl. Thereby, the establishment of a chemiosmotic potential between stroma and lumen of the chloroplasts (a major function of thylakoid membrane) can hinder by steric effects induced by chemical bond of protons on the extremely complex, highly organized transmembrane proteins (see Fig. 1). This effect should also lead to an inhibition of the so-called proton-pump in thylakoid membrane and consequently to a reduction of the chemiosmotic potential, ATP-synthesis and the pH-value (pH 8) in the stroma. Chlorophyll fluorescence measurements on intact maize and bean plants subjected to different TCA-Na concentrations via the soil/root pathway, showed that PSII function was markedly inhibited (Strauss et al., 2004). This finding was corroborated by data obtained by working with isolated bean thylakoids (Smit, 2006) demonstrating a marked inhibition of PSII function, measured as a decreased O_2 evolution rate, upon addition of TCA. A reduction of physiological pH-value in the stroma (pH 8) has negative influence on activity of Rubisco (maximum activity at pH 8), the key enzyme for carboxylation of ribulose-1,5-bisphosphate by CO_2 in the Calvin–Benson cycle. In this connection, Strauss et al. (2004) reported a reduction of Rubisco activity in bean leaves (*Phaseolus vulgaris*) after application of TCA-Na via *soil/root* pathway. This led to the assumption that the TCA-anion is decarboxylated to $CHCl_3$ ($COCl_2$) as well as shown in (R.5) (R.6a) (R.6b) (R.6c) (R.7) and finally to HCl (R.8). The biodegradation of PER and the implication of its degradation products are schematically presented in Fig. 1.

In support of the above, Lange et al. (2004) reported on the phytotoxic effects of atmospheric PER on the efficiency of photosynthesis and electron transport in leaves of birches (*Betula pendula* ROTH) and needles of Scots pines (*Pinus sylvestris* L.). Additional information on inhibition of photosynthesis by the chlorohydrocarbons PER, $CHCl_3$ and hexachloroethane (C_2Cl_6), applied via the *soil/root* pathway to the steppe plant *Artemisia lerchiana,* has been obtained by Weissflog et al. (2006).

6. Conclusions

It may be concluded that

- Under drought stress PER is degradated to phytotoxic TCA by oxidative biodegradation in the thylakoids membrane after uptake by the plant via the *air/leaf (needle)* pathway (see also putative reaction pathways (R.1) (R.2) (R.3) (R.4).
- The formed TCA is most probably mineralised to CO_2 and HCl in the chloroplasts (stroma) by a variety of degradation routes (see reaction pathways (R.5) (R.6a) (R.6b) (R.6c) (R.7) (R.8) (R.9)).
- TCA and its metabolites formed in leaves prompt protein protonation on account of its high degree of dissociation.

This probably leads to structural and functional changes, e.g., in stability, structure and function of chloroplast membranes and enzymes.

- Because of its high degree of dissociation, the formed HCl inside the chloroplasts contributes to a pronounced increase in proton concentration in the stroma, ultimately interferes with the proton gradient over the thylakoids.
- ATP-synthesis and the Calvin–Benson cycle are negatively influenced by decreased pH in the stroma following the additional protonation during the oxidative biodegradation of PER.
- In addition to the above, destabilisation of the membrane potential of the exposed thylakoids might be evoked as a result of the physiological effects of the TCA-anion, leading to interference with the photosynthetic electron transport system.
- One PER molecule induces the chronologically separated appearance of at least five protons as a result of its oxidative biodegradation and hydrolysis of its metabolites in chloroplasts. The protons in turn generate a clearly negative effect on the physiological status of the plant, depending on the number of PER molecules transformed.

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