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Measurement of Ion Leakage from Plant Cells in Response to Aquatic Pollutants

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In order to estimate the ecological impact of a man-made compound released into the environment it is necessary to measure its toxicity for various species from various levels of biological organization (Cairns 1983). Conventional tests, that determine the toxic potential of such compounds are mostly restricted to single species or a small group of organisms. Inferences on the toxicological data resulting from these tests, e.g. the *Daphnia* test, are safely transferable only to closely related organisms. The plasma membranes of all organisms are primary targets in the attack of environmental pollutants. With the aim of establishing a test based not on a single species but on essential properties of all organisms, we developed a non-invasive method of monitoring membrane integrity. The described method determines ion leakage in plant cells. Determining ion leakage is already a common method of assessing the extent of plant cell damage resulting from various types of stressors such as ozone (McKersie et al. 1982), chilling (Murray et al. 1989), and herbicides (Yanase et al. 1990).

We improved the conventional ion leakage assays in a way which allows for the continuous measurement of electrical conductivity in the medium of plant cells in suspension culture. Using this method, we have investigated the membrane toxicity of compounds from three important groups of anthropogenic pollutants in aquatic systems (Watts and Moore 1988; Gulyas et al. 1993). Group A includes substances that cause membrane disintegration by reactive radicals. These substances are introduced into aquatic systems in the form of peroxide-based bleaches and in the form of herbicides whose action is based on the production of reactive radicals (Summers 1980). Group B includes organic solvents that interact with membrane lipids because of their nonpolar nature. Group C includes detergents that damage membranes by integrating in the lipid bilayer and by solubilizing membrane constituents (Marcomini et al. 1988). These substances are major components of various cleaning agents. The effects observed for plant cells demonstrate the versatility of the test system with respect to various types of toxicants, indicating the suitability of the method as a biotest for monitoring aquatic pollution.

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MATERIALS AND METHODS

Tobacco (*Nicotiana tabacum* L. cv. Bel B) cell suspension cultures, donated by Ch. Langebartels (Neuherberg, Germany) were grown in LS-medium (Linsmaier and Skoog 1965). Composition (mg L^{-1}): KNO_3 (1900), NH_4NO_3 (1650), $MgSO_4 \cdot 7H_2O$ (370), $CaCl_2 \cdot 2H_2O$ (440), KH_2PO_4 (170), $MnSO_4 \cdot 4H_2O$ (22.3), KI (0.83); H_3BO_3 (6.2), $ZnSO_4 \cdot 7H_2O$ (8.6), $CuSO_4 \cdot 5H_2O$ (0.025), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $CoCl_2 \cdot 6H_2O$ (0.025), $FeSO_4 \cdot 7H_2O$ (27.86), Na_2EDTA (37.26), myo-inositol (100), thiamine-HCl (0.4), sucrose (30,000), 2,4-dichlorophenoxyacetic acid (1.0), and kinetin (0.2). The pH was adjusted to 5.8 with KOH before addition of the hormones. All substances were purchased in p.A. quality from Merck (Darmstadt, F.R.G.). Every 7 d the cells were subcultured in 40 mL of LS-medium in 250-mL Erlenmeyer flasks. The flasks were agitated in the dark at 120 rpm in a 5-cm orbit at 25°C.

For the measurement of ion leakage, cells from the late exponential growth phase (generally 7-d old cultures) were collected by gentle suction-filtration and washed three times with 40 mL per flask of "electrical conductivity-medium" (EC-medium). EC-medium consists of salts in 1/100 of the concentration of LS-medium. In addition, sucrose was replaced by 100 m*M* sorbitol. Washed cells (4 g) were transferred to 40 mL of EC-medium in a 250-mL Erlenmeyer flask. After 4-5 hr, stressors classified according to three groups were added:

- **Group A** (radical producing agents): *t*-butylhydroperoxide p.A. (Fluka, Buchs, Switzerland), Paraquat p.A. (Serva, Heidelberg, F.R.G.)
- **Group B** (organic solvents): methanol p.A., ethanol p.A., 1-propanol p.A., 2-propanol p.A., dimethyl-sulfoxide p.A. (DMSO), acetone p.A. (all from Fluka, Buchs, Switzerland)
- **Group C** (detergents): Tween 20 (Polyoxyethylenesorbitanmonolaurate) Tween 40 (Polyoxyethylenesorbitanmonopalmitate) Tween 60 (Polyoxyethylenesorbitanmonostearate) Tween 65 (Polyoxyethylenesorbitantristearate) Tween 80 (Polyoxyethylenesorbitanmonooleate) Tween 85 (Polyoxyethylenesorbitantrioleate) (all from Merck, Darmstadt, F.R.G.).

The experimental setup used to measure the electrical conductivity in the medium of the cell suspensions is shown in Figure 1. A small portion of the medium was continuously pumped through a flow conductivity cell and returned to the flask. The medium was passed through a nylon net which retained the cells in the flask. The total volume of medium in the measuring system was about 4 mL. During conductivity-measurement the flasks were rotated at 120 rpm in a 5-cm orbit at 25°C in the dark. For the simultaneous measurement of several samples a microcontrol was used to switch the conductivity meter at 150-sec intervals from one electrode to the next. Eight flasks were measured simultaneously.

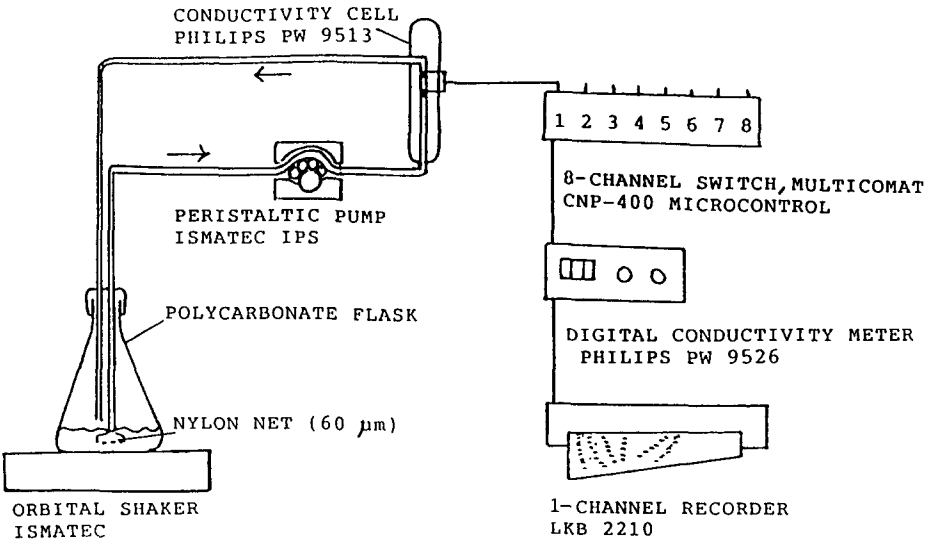

Figure 1. Experimental setup for the measurement of conductivity in plant cell suspension cultures. The cell suspension medium is pumped continuously through a flow conductivity cell. The conductivity is measured simultaneously in eight samples.

RESULTS AND DISCUSSION

Continuous measurement of conductivity in a tobacco cell suspension medium was achieved by pumping the medium through a flow conductivity cell (Fig. 1). Background conductivity was reduced by setting the ion strength of the test-medium (EC-medium) at 1/100 of LS-medium. A reduction of the uptake of ions by metabolically active cells was achieved by replacing sucrose with sorbitol, which is poorly metabolized by tobacco cells (Dracup et al. 1986).

Figure 2 shows the rate of conductivity in the cell culture medium upon addition of the stressors *t*-butylhydroperoxide (*t*-BuOOH) and Paraquat. The medium of untreated controls showed an initial increase in electrical conductivity followed by a decrease reflecting the uptake of ions from the medium by the cells.

t-BuOOH, which has been shown to induce membrane damage in plants (Koch et al. 1993; Matsuo et al. 1989), caused a rapid, concentration-dependent increase in conductivity (Fig. 2A). Since the variability of the rate of conductivity between identically treated samples was very small, the method should allow the detection of minute effects. Maximal conductivity values after treatment with relatively high concentrations of *t*-BuOOH did not further increase after freeze-thaw treatment of the cultures and were in the same range as those measured after freeze-thawing of untreated cultures. Hence, the

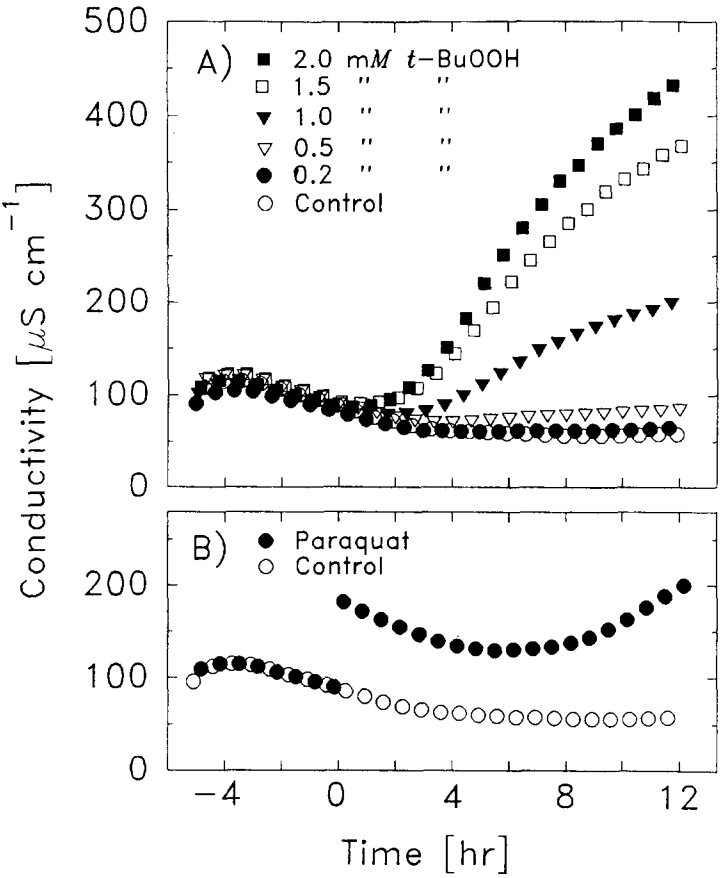

Figure 2. Effect of A) *t*-butylhydroperoxide (*t*-BuOOH) and B) Paraquat on the conductivity of the medium of tobacco cell suspension cultures. The cells were transferred to EC-medium, and 5 hr later (time zero) *t*-BuOOH (final concentrations between 0.2 m*M* and 2 m*M*) or Paraquat (0.5 m*M*) were added. Only every 16th data point is shown.

observed increase of conductivity during *t*-BuOOH-treatment was due mainly to an efflux of ions caused by membrane damage rather than to a metabolic response of the cells to *t*-BuOOH or to generation of ions by the reaction of *t*-BuOOH with cellular components.

t-BuOOH also led to a concentration-dependent inhibition of cell culture growth (Fig. 3). Just as observed for ion leakage (Fig. 2A), 0.5 m*M* *t*-BuOOH showed a marked effect, and 0.2 m*M* *t*-BuOOH showed only little but significant effect. Moreover, *t*-BuOOH caused an increase of thiobarbituric acid reactive substances resulting from degradation of the fatty acid components of the plasma membrane (data not shown). These observations further confirm the suitability of our system as a test of membrane integrity and cell viability.

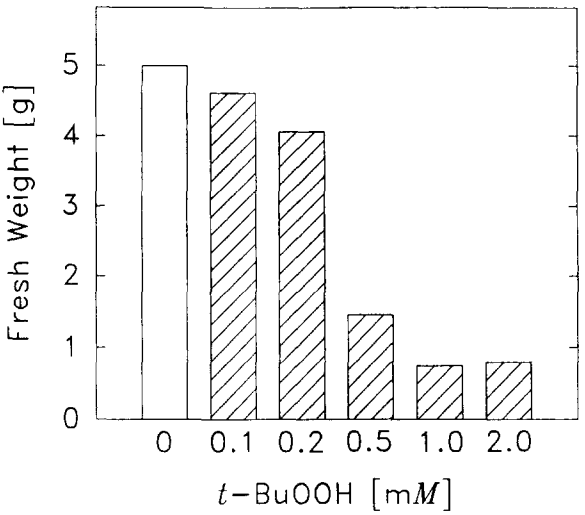

Figure 3. Effect of t-butylhydroperoxide (*t*-BuOOH) on the fresh weight of tobacco cell cultures. *t*-BuOOH was added immediately after subculturing 1.5 g (fresh weight) of cells. Seven days later the cells were separated from the medium by suction-filtration and fresh weight was determined.

Figure 2B shows the effect of Paraquat on the conductivity of the cell suspension medium. Paraquat caused an initial conductivity increase due to its ionic nature. Ion leakage, however, was observed only after a lag phase of several hours. In contrast to *t*-BuOOH, which provokes oxidative damage to the cells from outside, Paraquat catalyzes the formation of reactive oxygen species within the cell (Summers 1980).

The initial decrease of conductivity during the treatment with Paraquat can be explained by an uptake of the herbicide, while the following increase shows the radical-induced damage. Thus, the different rates of conductivity in the case of cell cultures treated with *t*-BuOOH and Paraquat probably reflect the different mechanism of action for each substance.

The kinetics of conductivity in Paraquat-treated cell cultures (Fig. 2B) demonstrates that continuous measurements, as recommended by Whitlow et al. (1992), are preferable to using only one or a few points in time for the determination of ion leakage. Paraquat uptake and ion leakage have opposite initial effects on conductivity. Thus a single measurement after the addition of Paraquat may lead to erroneous conclusions.

In addition, the toxic effects of various alcohols and other organic solvents have been examined (Fig. 4). At a concentration of 1 % (v/v), methanol,

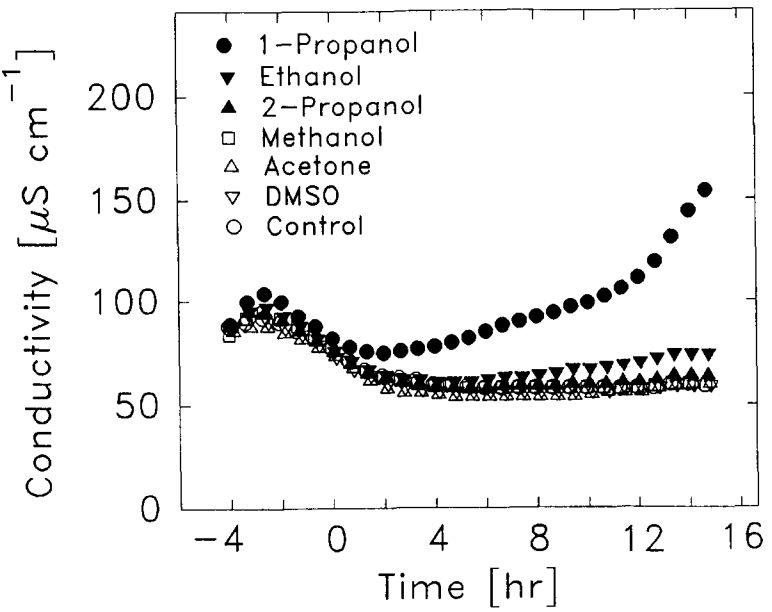

Figure 4. Effect of solvents on the conductivity of the medium of tobacco cell suspension cultures. The cells were transferred to EC-medium, and 4 hr later (time zero) the solvent was added in a final concentration of 1 % (v/v). Only every 16th data point is shown.

2-propanol, acetone or dimethylsulfoxide (DMSO) caused no increase in medium conductivity. Ethanol induced a small but significant increase while 1-propanol was found to be very toxic.

Toxicity results for various detergents of the Tween series have also been compared (Fig. 5). At a concentration of 1 % (v/v), Tween 20 led to a steep increase of conductivity, while Tween 40, 60, 65, 80 and 85 showed no membrane damage. Tween 20, containing a C_{12} (lauric acid) fatty acid, may disturb the arrangement of fatty acids in the plasma membrane because of the short chain length of its nonpolar residue. The fatty acids of the plasma membrane, however, mostly range from C_{16} (palmitic acid), to C_{18} (stearic acid, oleic acid, linoleic acid, linolenic acid) and C_{20} (arachidonic acid), which is similar to the range of fatty acids in Tween 40 to 85 (C_{16} to C_{20}). This example shows that the method can be useful to determine structure-activity relationships for specific toxicants.

Our study results show that the method we have developed is suitable for ascertaining the membrane toxic effects of various pollutants in aquatic systems. The induced membrane damage and subsequent ion leakage is likely to represent the destruction of basic membrane elements, which are common to all cellular organisms.

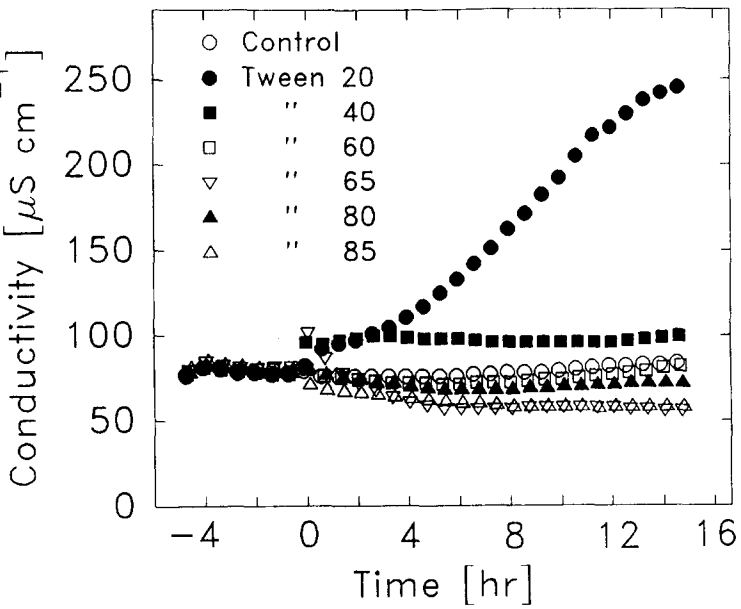

Figure 5. Effect of Tween detergents of varying length on the conductivity of the medium of tobacco cell suspension cultures. The cells (2 g) were transferred to EC-medium and 4 h later (time zero) the detergent was added in a final concentration of 1 % (v/v). Only every 16th data point is shown.

Inferences as to the effects that membrane-toxic compounds cause in tobacco cells should also be observed in other plant, animal and bacterial systems. Thus, it should be possible to assess the impact of membrane-toxic compounds on an ecosystem by testing different organisms from different trophic levels of that ecosystem in one single standardized test. Experiments with other organisms and dose-effect studies with various concentrations of toxic compounds are currently underway to establish that the continuous measurement of ion leakage is a viable biotest for monitoring membrane toxic compounds in waste water or recipient streams.

In conclusion, our method allows the noninvasive and continuous monitoring of membrane integrity in plant cells. The observed effects of ion leakage in response to reactive radicals, organic solvents and detergents exemplifies the suitability of the test system for measuring membrane impairment by various membrane toxic compounds which are introduced into aquatic systems by anthropogenic pollutants.

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REFERENCES

Cairns J Jr (1983) Are single species toxicity tests alone adequate for estimating environmental hazard? Hydrobiologia 100:47-57

Dracup M, Gibbs J, Greenway H (1986) Melibiose, a suitable, non-permeating osmoticum for suspension-cultured tobacco cells. J Exp Bot 37:1079-1089

Gulyas H, Reich M, Eickhoff H-P, Holst H-J, Sekoulov I (1993) Identifizierung organischer Einzelsubstanzen in Abläufen biologischer Kläranlagen. Wasser Abwasser 134:486-491

Koch U, Glatzle D, Ringenbach F, Dunz T, Steger-Hartmann T, Wagner E (1993) Quantification of oxidative damage in plant cell suspension cultures by continuous monitoring of ion leakage. Physiol Plant 85 Part 2:A67 (Abstract)

Linsmaier E M, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100-127

Marcomini A, Capri S, Brunner P H, Giger W (1988) Mass fluxes of linear alkylbenzenesulfonates, nonylphenol, nonylphenol mono- and diethoxylate through a sewage treatment plant. In: Angeletti G, Bjørseth A (eds) Organic micropollutants in the aquatic environment. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 266 (Abstract)

Matsuo T, Kashiwaki Y, Itoo S (1989) Membrane damage caused by exposure to t-butylhydroperoxide. Phytochemistry 28:1003-1006

McKersie B D, Hucl P, Beversdorf W D (1982) Solute leakage from susceptible and tolerant cultivars of *Phaseolus vulgaris* following ozone exposure. Can J Bot 60:73-78

Murray M B, Cape J N, Fowler D (1989) Quantification of frost damage in plant tissues by rates of electrolyte leakage. New Phytol 113:307-311

Summers L A (1980) The bipyridinium herbicides. Academic Press, London

Watts C D, Moore K (1988) Fate and transport of organic compounds in rivers. In: Angeletti G, Bjørseth A (eds) Organic micropollutants in the aquatic environment. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 154 (Abstract)

Whitlow T H, Bassuk N L, Ranney T G, Reichert D L (1992) An improved method for using electrolyte leakage to assess membrane competence in plant tissues. Plant Physiol 98:198-205

Yanase D, Andoh A, Yasudomi N (1990) A new simple bioassay to evaluate photosynthetic electron-transport inhibition utilizing paraquat phytotoxicity. Pestic Biochem Physiol 38:92-98