

Adaptation of the Pollen Tube Growth Assay to Cytotoxicity Testing of Cigarette Smoke

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Summary

We describe a recently developed *in vitro* assay for the assessment of the cytotoxicity of gaseous materials. The method is based on the pollen tube growth (PTG) test suited to determine the cytotoxicity of non-gaseous, water-soluble substances (Kristen and Kappler, 1995). Instead of the conventional PTG test, we used a semi-automatic, computer-controlled multi-channel device for the injection of gases and gas mixtures into a battery of incubation tubes containing germinating, suspension-cultured tobacco pollen as indicator of toxic effects. The efficiency of the new PTG gas test device was examined using the gas phase of cigarette smoke. The examination exhibited sufficient sensitivity of the method and high reproducibility of the toxicity data obtained. The assay can be adapted to various sources of exhaust gases.

Key words: *In vitro* toxicology – Cigarette smoke – Biotechnology – Pollen test

Zusammenfassung: Anpassung des Pollenschlauch-Wachstumstests an die Zytotoxizitätsprüfung von Zigarettenrauch.

Wir beschreiben ein kürzlich entwickeltes *in vitro* Verfahren zur Abschätzung der Zytotoxizität von gasförmigen Stoffen. Die Methode basiert auf dem Pollen Tube Growth (PTG)-Test, der für die Bestimmung der Zytotoxizität von nicht-gasförmigen, wasserlöslichen Substanzen geeignet ist (Kristen und Kappler, 1995). Anstelle dieses konventionellen PTG-Tests verwendeten wir eine halbautomatische, computergesteuerte Mehrkanal-Apparatur für die Injektion von Gasen in eine Batterie von Inkubationsgefäßen. Diese Gefäße enthalten als Indikator für die toxische Wirkung keimende Tabakpollen in Suspensionskultur. Die Wirkungsweise und Effizienz dieser neuen PTG-Gastest-Apparatur wurde unter Verwendung der Gasphase von Zigarettenrauch geprüft. Die Untersuchungen zeigten, daß die Empfindlichkeit des Verfahrens ausreicht, um gut reproduzierbare Ergebnisse zu liefern. Der PTG-Gastest kann an verschiedene Abgasquellen angepaßt werden.

1 Introduction

The pollen tube growth (PTG) test is one of the most frequently used *in vitro* methods for the toxicity assessment of water soluble substances. It is based on the growth inhibition of *in vitro* cultivated tobacco pollen tubes (Kappler and Kristen, 1987), and was successfully applied for toxicity testing of herbicides (Strube et al., 1991), detergents (Kristen et al., 1993), drugs (Barile et al., 1994) and cosmetic formulations (Gettings et al., 1994). The PTG test can also be used for screening of gaseous substances after modification of the incubation technique, whereas the majority of other available assays is not suited for this purpose.

In the PTG gas test, the gases are injected into gas tight incubation tubes fixed on a multi-channel incu-

bation device. Thus, the screening procedure can be semi-automatically performed using the gas phase of a cigarette smoking device.

In the following, the equipment and the procedure of the PTG gas test is described. Moreover, technical results of the method are presented and discussed confirming the suitability of the test to discriminate between the inhibitory potentials of the gas phases obtained from different cigarette types.

2 Materials and Methods

Pollen of tobacco plants (*Nicotiana glauca* L. and *Nicotiana glauca* L.), collected prior to anthesis, air-dried and stored at minus 20° C, was used for the experiments. The pollen culture medium consisted of double-distilled

water, containing 10% sucrose (w/v), 3 mM Ca(NO₃)₂ and 0.01% boric acid (w/v), and 10 mM MES (2-[N-morpholino]-ethane-sulfonic acid) (Serva, Heidelberg, Germany). The medium was adjusted to pH 5.6 with KOH and frozen for storage. An ethanolic Alcian blue stock solution, stable for some months in the dark, was prepared by dissolving 0.5 g Alcian blue 8GX (Sigma, Deisenhofen, Germany) in 100 ml absolute ethanol.

Tobacco pollen was suspended in the culture medium, and the suspension, while producing pollen tubes, was incubated with the test material for 18 h at 27° C in the dark (Kappler and Kristen, 1987).

The Alcian blue staining method for photometric quantification of pollen tube mass production was used according to a procedure previ-

ously reported by Kappler and Kristen (1988). Alcian blue binds to water-insoluble polysaccharides of the pollen tube walls when added to the suspension after the 18 h incubation period. After several washings of the pollen tubes, the dye was redissolved by acidification with citric acid and quantified photometrically in the supernatant. The extinction values have been shown to linearly correlate with the amount of pollen tube material produced (Kappler and Kristen, 1988).

In the conventional PTG test, used for solid and liquid water-soluble test substances as recently described in detail (Kristen and Kappler, 1995), the procedures of incubation, staining and washing were performed in single incubation flasks and test tubes. For gases and gas mixtures, these procedures were run in a multi-channel filter device developed to shift the test from manual to computer-controlled, semi-automatic operation.

The multi-channel filter device consists of incubation tubes inserted in a cube-shaped plexiglas frame construction (fig. 1). The size of this so-called filter block and the ar-

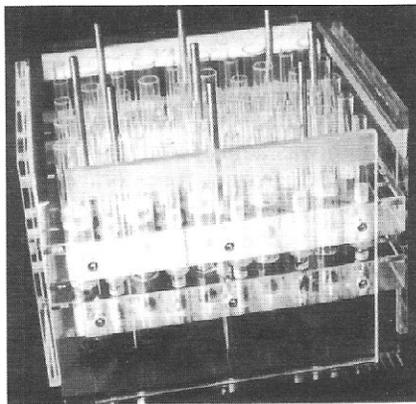


Figure 1: The filter block (a multi-channel filter device) consists of 50 incubation tubes inserted in a cube-shaped plexiglass frame construction. Each of the 10 rows has 5 incubation tubes with different volumes for simultaneous incubation with 5 different gas quantities. The bottoms of the tubes are closed by a rubber gasket pressed to the tube openings by a thick, screw-fixed plexiglass plate.

rangment of the incubation tubes fit exactly the pattern of commercial photometer vessel plates. There are 5 different tube volumes in the filter block. Thus, it is possible to use 5 different gas quantities simultaneously in each experiment. Both ends of the incubation tubes are open but can be closed for gas tightness by rubber gaskets pressed to the openings by thick screw-fixed plexiglas cover plates. The test gases were injected through the rubber septum into the incubation tubes containing the pollen suspension.

The correct function of the filter block was examined by the use of gas phases of cigarette smoke. The source of the gas phases, obtained from different cigarette types, was a single-channel smoking apparatus (Borgwaldt Co., Hamburg, Germany) kindly provided by the B.A.T. Cigarettenfabriken GmbH in Hamburg. This apparatus simulates cigarette smoking puff by puff, filters the smoke of each puff in order to retain the particle fraction, and releases the pure gas phase into the test device. The gas phase of one or two defined puffs was then sucked into a reservoir consisting of two 50 ml glass syringes. From there, defined amounts of gas were injected into the incubation tubes of the filter block by a multi-channel injector. The flow of gas from the exit of the smoking apparatus to the incubation tubes is driven by a vacuum pump which gives rise to an exactly defined amount of negative pressure within the tubes. This negative pressure is compensated for from the gas of the reservoir at one go causing a synchronous filling of the incubation tubes with volume-related aliquots of the test gas. The vacuum pump was controlled by a personal computer. The whole device is schematically presented in figure 2.

In order to produce a dose-response curve of a certain test material, each row of incubation tubes is supplied with different amounts of the test gas. After the 18 h incubation period, one of the two rubber gaskets closing the incubation tubes was

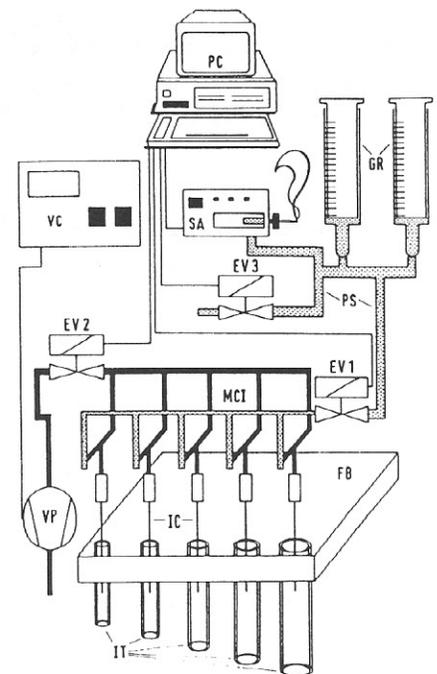


Figure 2: Schematic drawing of the complete device used for the PTG gas test. The instruments, vessels and pipes are marked as follows: PC = personal computer controlling the production, storage, flow and injection of the test gas. SA = one-channel smoking apparatus producing the gas phase of cigarette smoke puff by puff. GR = gas reservoir (two 50 ml syringes) storing the gas phase before injection into the incubation tubes. FB = filter block (see also fig. 1) containing the incubation tubes (IT) with different volumes (both ends of the tubes are closed during incubation). MCI = multi-channel injector for gas injection into the incubation tubes through a silicon septum. IC = injection cannules. VC = vacuum controller keeping a constant partial vacuum within the gas pipe system (PS) connecting the smoking apparatus with the incubation tubes. VP = vacuum pump. EV1-3 = electromagnet valves for control of the gas flow (EV1), for adjustment of the partial vacuum (EV2), and for ventilation of the gas pipe system (EV3).

exchanged for a filter plate mainly consisting of a Nylon sieve with a mesh size of 20 μm . This mesh size is suited to retain the pollen tubes when the culture medium is sucked off from the incubation tubes. The following procedures, staining with Alcian blue, removal of the dye surplus, washing of the stained pollen tubes, and redissolvement of the

bound dye by acidification with citric acid were performed according to the procedures of the conventional PTG test (Kristen and Kappler, 1995). However, the centrifugation steps of the conventional test and the transfer of the supernatant, containing the redissolved Alcian blue, were replaced by sucking off the fluids through the Nylon sieve using a vacuum pump. Thereby, the dye solution was directly sucked into the photometer vessels. This step was possible because of the adjustment of the filter block to commercial photometer plates. Finally, the extinctions of the Alcian blue solutions were measured at 607 nm using a Beckman photometer (model 34).

Concentration effect curves were established by plotting the injected gas amounts (ml) against the percentage of pollen tube growth inhibition. The IC_{50} values were extrapolated from the curves using linear regression analysis as reported earlier (Kappler and Kristen, 1987, 1988).

3 Results and Discussion

In order to optimize the efficiency and reproducibility of the described PTG gas test method, some systemic parameters, concerning the five following questions, had to be examined: (1) does the biomass (amount of pollen per ml culture medium) influence the growth inhibition, (2) does the degree of growth inhibition depend on the concentration or on the quantity of gas mixture injected, (3) which role plays the amount of culture medium filled into the incubation tubes, (4) is there an „ageing“ of the gas mixture during its storage, and finally (5), does test gas residue, possibly sedimenting within the test device, influence the results?

To examine the first question, four different pollen concentrations and three different test cigarette types were used as gas sources. As shown in figure 3, there exists a linear relationship between the suspended biomass and the achieved IC_{50} val-

ues, i.e., the pollen tube growth inhibition decreases linearly with increasing amounts of pollen present in the incubation tubes. This result is surprising because similar tests using ethanol and dinitrophenol as growth inhibitors did not show a linear correlation. However, it cannot be answered yet whether this regularly observed linearity for gases and gas mixtures results from an active detoxification or a passive absorption or both by the pollen suspension. Therefore, unique pollen material with constant germination and growth rates should be used for each experiment of an experimental series.

A second series of test experiments using a filter block with different volumes of the incubation tubes showed that the IC_{50} values depend on the absolute quantity of the test gas present in the reaction space of an incubation tube (space not occupied by the pollen suspension). The amount of pollen suspension with constant pollen concentration plays no considerable role in regard to the reaction of the test system. As shown by figure 4, the IC_{50} value barely

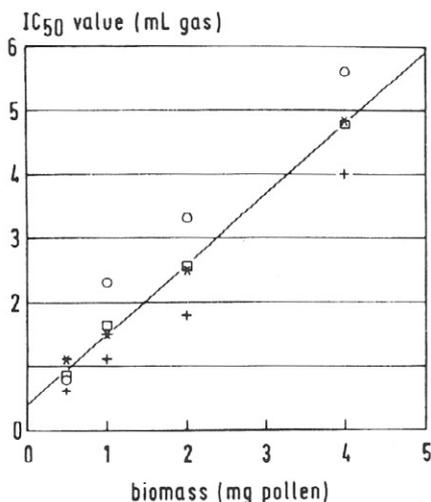


Figure 3: Linear correlation between IC_{50} values (mean inhibitory gas quantities) and biomasses (amounts of pollen in mg per ml culture medium) used in the experiment. Gas phase was taken from 3 different test cigarettes (o = type I, * = type II, + = type III, □ = mean).

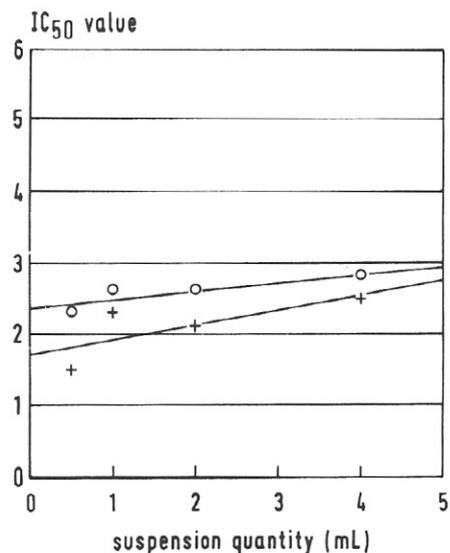


Figure 4: Dependency of the IC_{50} values on the quantities of culture medium with constant amount of standard cigarette gas phase. As shown for two experiments (o and +), pollen tube growth inhibition does not significantly decrease with increasing quantity of culture medium.

increases with a higher suspension quantity.

The influence of test gas ageing was investigated by storing of the gas phase of freshly exhausted cigarette smoke for 1 min up to 170 h before injection into the incubation tubes. In figure 5 the decrease of toxicity with ageing of the gas phase is clearly exhibited. During the first 6 h after the exhaust of a cigarette puff, the IC_{50} of the stored gas phase rises steeply reflecting dramatic reduction of the inhibitory effect to the growth of pollen tubes. Later, growth inhibition decreases slower. Toxicity reduction during ageing implements that the most reactive (i.e. most toxic) constituents of the gas phase are progressively decontaminated by chemical reaction with other components, by condensation or by sedimentation.

Sedimentation of inhibitory components of the gas phase was indirectly observed when several cigarettes were successively smoked without cleaning of the pipe system of the device. As demonstrated by figure 6, the IC_{50} of a puff decreased with increasing number of cigarettes

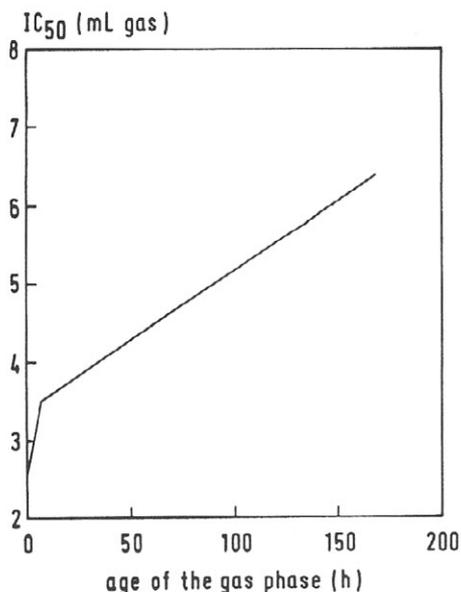


Figure 5: Influence of gas ageing on the inhibitory effect of the cigarette gas phase. The IC_{50} rises dramatically within the first 6 h of gas storage (i.e. very strong ageing in this period).

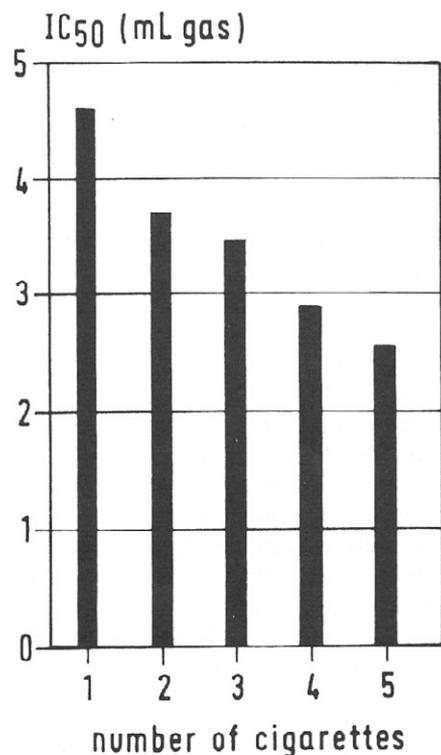


Figure 6: Influence of the number of successively smoked cigarettes (5 puffs per min each) on the IC_{50} values of the gas phase of a puff following 1, 2, 3, 4 or 5 cigarettes, respectively. The inhibitory effect of the gas phase increases with increasing number of cigarettes smoked prior to the test puff.

smoked before cleaning. This effect was probably caused through vaporization of previously sedimented components by the fresh and hot gas stream of a following puff. Rinsing with ethanol of the gas pipes in the smoking device reduced this effect approximately to zero.

4 Conclusion

The examinations of the inhibitory effect of gas phases obtained from cigarette smoke revealed interesting and important aspects of *in vitro* toxicity assessment of gas mixtures. The results show that the described semi-automatic test device is working very satisfactorily when some systemic parameters are carefully considered, i.e., very rapid ageing of the gas mixture, sedimentation of inhibitory components and their reactivation by a fresh gas stream, and the exact control of the gas quantity injected into the incubation tubes.

The central part of the test device, the so-called filter block, was shown to function as a multi test tube system which itself enables a reduction of the test duration to about 50 % in relation to the conventional PTG test. However, the main advantage of the PTG gas test device is not the saving of time but the parallel and synchronous testing of 5 different volumes of the same test gas sample.

Taken together, the PTG gas test device produces toxicity data of gas mixtures with high reproducibility and sensitivity. It is well suited for cytotoxicity screening of gaseous test materials on the base of a plant-derived, non-animal *in vitro* system.

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