The method of *Allium* anaphase–telophase chromosome aberration assay

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Emissions of genotoxic chemicals from anthropogenic activities into environmental compartments require genotoxicity assays for the assessment of the potential impact of these sources on the ecosystems. The *Allium* anaphase–telophase chromosome aberration assay has been developed as a method for rapid screening of chemicals and environmental samples. For determination of sample concentrations prior to genotoxicity testing, a 96-h root growth inhibition test is carried out. In the chromosome aberration assay, root tip cells are investigated after a 48-h exposure. Bridges and fragments are scored as indicators of clastogenicity, and laggards or vagrant chromosomes are considered indicators of spindle poisoning. The assay is simple and reliable and can be used for genotoxicity studies of wastewater, river water, contaminated soils and other complex mixtures.

Key words: *Allium cepa*, genotoxicity, chromosome aberration, anaphase–telophase, complex mixture

INTRODUCTION

Genotoxic chemicals used for many purposes in manufacturing processes can be found in environmental compartments such as air, water, soil and sediments. The chemicals can enter the environment from discharged wastewater, air emissions, during consumption of the products and from domestic and industrial waste sites.

For evaluation of environmental samples, many genotoxicity assays are used; among these, the *Salmonella* mutagenicity assay is the most commonly applied test system for complex mixtures (Claxton et al., 1998). However, many plant assays have also appeared to be useful and are in some ways superior compared to the *Salmonella* test. Plants are often more sensitive to heavy metals (Fiskesjö, 1988) than the *Salmonella* strains; moreover, it is possible to expose plants directly to complex mixtures or environmental samples either in the laboratory (Fiskesjö, 1985) or *in situ* (Grant et al., 1992). The present paper describes the technical procedure of the *Allium* chromosome aberration assay, which was developed into a cheap and rapid screening test. The assay is a modification of the *Allium* test described earlier by Fiskesjö (1985). The test system was simplified so that only certain aberrations in the anaphase and telophase are scored.

OUTLINE OF THE TEST SYSTEM

The most important advantage of the *Allium* test is that it is a "low budget" method, which besides being fast and easy to handle also gives reliable results. The duration of the genotoxicity test is three to four weeks, including initial toxicity testing, scoring of aberrations, and statistics. It can be described briefly as follows:

Week 1: A 96-h root growth inhibition test is carried out in order to determine the toxicity level of the test chemical or environmental sample, and EC_{50} is determined by the dose–response relationship by interpolation.

Week 2: The 48-h genotoxicity test is carried out with 3–4 concentrations below the EC_{50}, and the root tip cells are prepared for microscopic analysis.

Week 3: Chromosome aberrations are scored in anaphase and telophase cells.

Week 4: Calculations, statistics and reporting.

MATERIALS AND CHEMICALS

The test organism

The common onion, *Allium cepa* (Stuttgarter Riesen) is used. In Denmark, onions can be obtained from Dähnfeldt, Odense. The onions are sown in

spring and harvested in late summer. The bulbs should be 15–22 mm in size and weigh 2–4 g. However, onions of other sizes and sorts can be used. If kept dry at 10–15 °C, the onions can be used within a year after harvest. The onions need to rest for about two to three months before they are able to grow roots fast enough for the assay. The yellow shallows and the dry bottom plate inside the root primordia are carefully removed prior to the test.

Glassware

Figure 1 shows some special equipment used for the *Allium* test. The glass tubes (Wallin-glass) are bottomless, and a 70-mm ruler is mounted on the side of the glass and then used to measure the length of the root bundle. The beakers are made of polycarbonate and are disposable. This equipment is produced at our own laboratory, but the assay can also be carried out using normal test tubes in a rack and an ordinary ruler for the measurement of the root length. However, the advantage of this special equipment compared to the test tubes is that it is easier to change the test solutions in the beaker. A hole in the cover makes it also possible to aerate the solution in the beaker, which is more complicated if six test tubes are used. One disadvantage of using one beaker instead of six test tubes is that the volume of test solution is about twice as large as for test tubes.

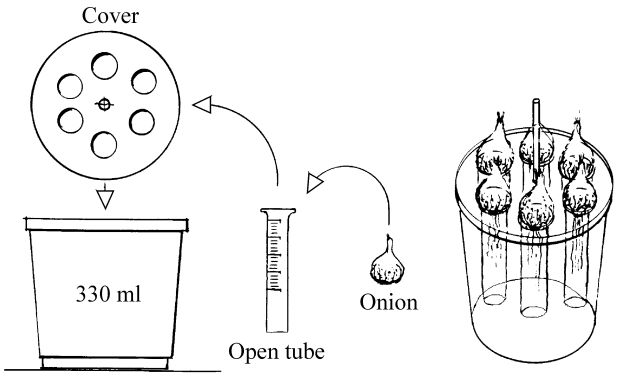

Fig. 1. Equipment for the exposure of onion roots in the *Allium* test (Rank 1997)

Chemicals

Tap water of good quality is used for negative control and for dilution of chemicals. Good quality means, for example, that the water is not containing any chlorine compounds and that the water pipes are not made of copper. If the quality of the tap water is poor, it is recommended to use synthetic fresh water made of Millipore water containing $MgSO_4$ 60 mg/l, $NaHCO_3$ 96 mg/l, KCl 4 mg/l and $CaSO_4$ 60 mg/l ($CaSO_4$ which should be dissolved by heating and stirring before it is mixed with the other salts). If the test chemicals are not water-soluble, DMSO, acetone or ethanol can be used as a solvent. Methyl methanesulfonate (MMS) can be used as positive control. Fixation and maceration is carried out using a solution of 9 parts of 45% acetic acid and 1 part of 1 M HCl. The chromosomes are stained with 2% orcein in 45% acetic acid.

Microscope and photo equipment

A light microscope (*e. g.*, Dialux from Leitz) is used with an oil immersion objective and 500× magnification. For discussion of aberrations, it is useful to install a video camera on the microscope and transfer the pictures to a computer.

METHODS

Prior to the *Allium* test the pH of an environmental sample (*e. g.*, wastewater) should be adjusted to about 7 with 1 M HCl or NaOH. The test is carried out at room temperature and the onions should be kept away from direct sunlight during the experiment.

Root growth inhibition test

The toxicity assay is performed as a 96-h semi-static exposure test, and 6–9 concentrations of the test chemical or complex mixture are used. Every 24 h the test solutions are replaced by fresh solutions. The test solutions are kept cold but should have reached room temperature before use. At the termination of exposure, one onion (out of six) with the poorest growth is discharged and the length of the root bundle is measured for the rest five onions. The effect of growth inhibition is shown on a graph with the log concentration against the root length expressed as percent of control (Fig. 4). The EC_{50} can be calculated with a computer programme or found by a simple interpolation.

Genotoxicity assay

The genotoxicity assay is carried out with four sample concentrations. They can, for example, be composed of the EC_{50} as the highest concentration followed by 50%, 25% and 10% of the EC_{50}. Tap water or synthetic fresh water can be used as a negative control, and if DMSO or other solvents are used, a solvent control should be included in the assay. MMS, 10 mg/l, is used as positive control, but maleic hydrazide, 5 mg/l, can also be used. Six onions are exposed to each concentration. For

the first 24 h, the onions are grown in tap water or synthetic fresh water, whereafter they are exposed to the test chemicals for 48 h, which is close to two cell cycles. As for the toxicity test, the test solution is changed after 24 h. The onion with the poorest growth is excluded for every concentration, and the remaining five onions are prepared for microscopy.

For every onion one slide is made using the following procedure: 5 root tips at a length of 10 mm are cut off and placed in a test tube with 2 ml acetic acid – hydrochloric acid solution and heated for 5 min at 50 °C. Thus, the root cells will become fixed and macerated. Thereafter, the roots are placed on a slide and the terminal root tips (1–2 mm) are cut off and used for further preparation. The rest of the material is removed from the slide and the excess of liquid is sucked up by a piece of blotting paper. Two drops of fresh filtrated 2% orcein solution are added and mixed with the root tips by stirring and knocking with a blunt stick of stainless steel (or something alike). In the final phase, a cover slip is placed on the root cells and allowed to absorb stain for 5–10 min. Afterwards, the cells are squashed by placing to layers of blotting paper on the cover glass and pressing slightly down with the thumb. The cover slip is fixed carefully to the slide with nail varnish. The slides can be kept fresh for 2 months in a freezer.

Microscopic examination

All slides are coded and examined blind. The microscopic analysis includes the mitotic index and scoring of chromosome aberrations in anaphase and telophase cells. The mitotic index, MI is found by counting all stages of mitotic cells out of 1000 cells. It is recommended only to score chromosome aberrations if MI is above 10, otherwise there will be too few anaphase and telophase cells for the analysis. The slides are examined from right to left, up and down, and the first 100 anaphase and telophase cells are scored for aberrations.

Two classical categories of aberrations, fragments and bridges, are scored. These are the most frequent aberrations and indicate that the test chemical or complex mixture is clastogenic. Very often fragments are seen together with bridges and these cells can be scored as a specific category. Figure 2 shows how chromatid breakage and rejoining can result in these aberrations. Categories as vagrant and laggards indicating interaction with the spindle are also scored. Other less frequent aberrations such as multipolarity, c-mitotic anaphases, polyploidy and pulverised chromosomes, are also registered. Different kinds of aberrations are shown in Fig. 3. The control level from exposure to tap water or synthe-

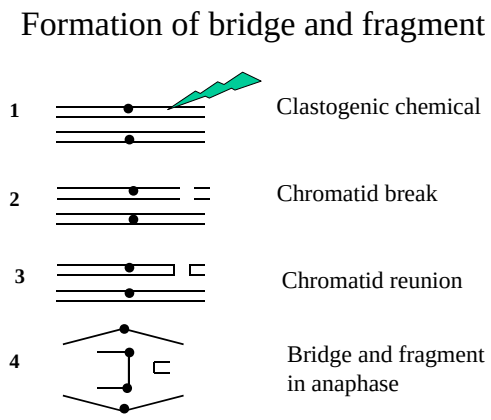

Fig. 2. The principle of chromatid breakage and rejoining during the mitosis resulting in the formation of a bridge and a fragment, which can se observed in the anaphase or telophase (after Kihlmann, 1971)

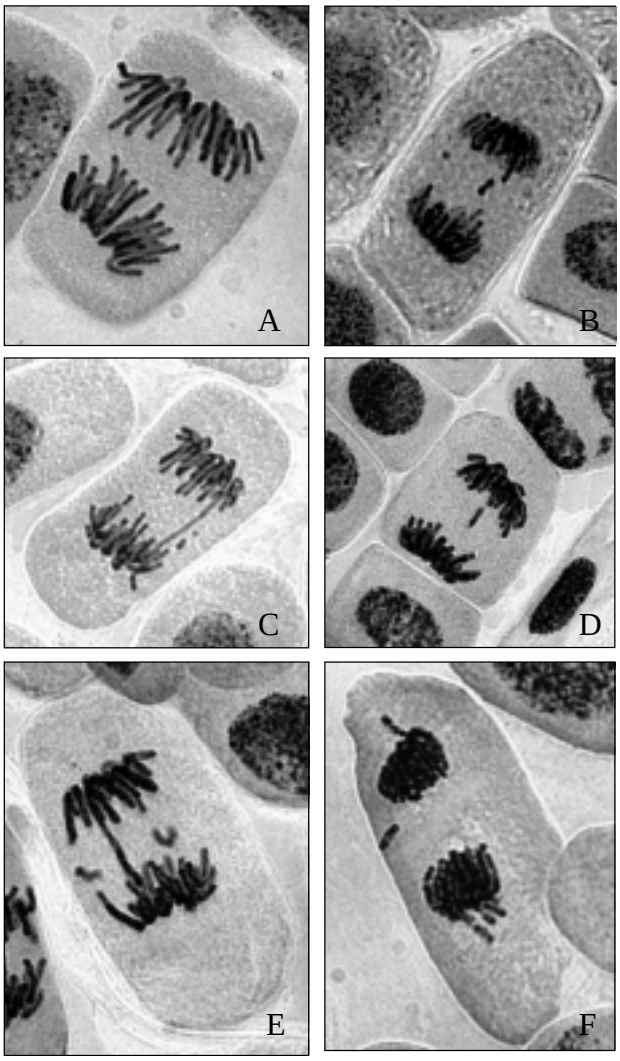

Fig. 3. The cells have been exposed for 48 h to maleic hydrazide, 5 mg/l. A: Normal anaphase cell. B: Small and big fragment. C: Bridge and two fragments. D: Laggard chromosome. E: Bridge + fragment and a laggard chromosome. F. Vagrant chromosome in telophase

tic freshwater is about 1% of aberrant cells, and for the positive control with MMS, 10 mg/l, or maleic hydrazide, 5 mg/l, it is about 25% of aberrant cells. If the toxicity is not too high, it is possible to score 100 anaphase and telophase cells per slide. With five onions per beaker it gives 500 cells per concentration. However, if the mitotic index is very low, it can be impossible to find 100 cells per onion.

Statistics

As the distribution of aberrant cells is binomial, we used the χ^2-test for statistical calculations. The calculations were carried out on the assumption that the five onions made one sample, and each concentration was tested against the control sample.

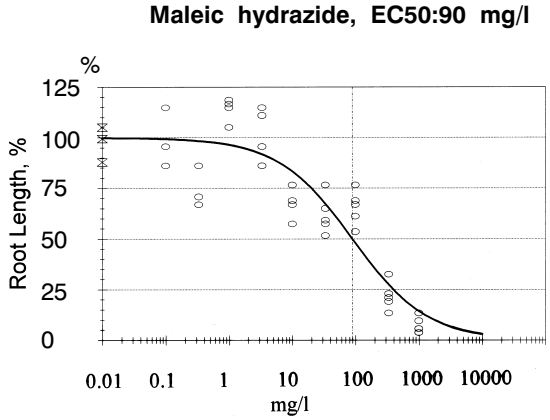

Fig. 4. Growth inhibition of *Allium* roots exposed to maleic hydrazide

RESULTS AND DISCUSSION

The results from a 96-h root growth inhibition test of maleic hydrazide are shown in Fig. 4. The sigmoid graph is a typical dose–response curve for this kind of toxic effects. However, if the chemical or environmental sample has a low acute toxicity, it can be difficult to obtain a usable graph for determination of EC_{50}. From earlier studies (Nielsen, 1994; Rank et al., 1994; Rank et al., 1998), EC_{50} values from testing chemicals, wastewater and wastewater sludge are shown (Table 1).

Table 2 shows the corresponding results from negative and positive controls. When tap water was used as negative control (Rank et al., 1997), 0.2% and 1.4% of aberrant cells were found, and for synthetic freshwater (Rank et al., 2002) the values were 0.4% and 1.6%. The present results for MMS, 10 mg/l, varied from 18.4% to 28.6%, but in other studies we have seen levels lower than 10% of aberrant cells. The explanation for these variations is that MMS degrades very fast, even if it is kept cold, and therefore it is recommended not to use a batch for more than half a year after it is opened.

In Table 2 one can see that MMS, 10 mg/l, decreased the mitotic index (9–21%) compared to the controls. Further, a big variation (42–65) can be seen for the MI of the controls. Therefore, it could be questioned if the MI should be used as a quantitative measure of toxicity, and in the *Allium* anaphase–telophase chromosome aberration assay the

Table 1. **Root growth inhibition expressed as EC_{50} of *Allium* roots exposed to chemicals, wastewater and wastewater sludge**

Media		EC_{50}
Chemicals, µM	Sodium chromate	143
	Formaldehyde	375
	1,1,1-Trichloroethane	2100
Wastewater, %	Municipal	>100
	Chemical plants	44–70
	Petrochemical industry	14
	Pulp- and paper mill	0.6
Wastewater sludge, g / l (dry weight)		0.23–22

Table 2. **Mitotic index and chromosome aberrations for corresponding control (tap water or synthetic fresh water) and positive control, MMS, 10 mg/l. Results from 4 separate assays**

	M.I. (± SD)	Chromosome aberrations per 500 cells				% Aberrant cells (± SD)
		Bridge	Fragment	Vagrant	Other	
Tap water	50 (15)	0	1	0	0	0.2 (0.4)
MMS	44 (3)	28	104	11	0	28.6 (12.9)
Tap water	53 (10)	4	2	1	0	1.4 (1.0)
MMS	48 (13)	38	78	13	0	25.8 (4.5)
Synthetic water	65 (17)	1	3	4	0	1.6 (0.9)
MMS	49 (15)	38	42	8	1	18.4 (4.1)
Synthetic water	52 (30)	1	4	0	0	0.4 (0.5)
MMS	42 (6)	50	66	10	0	25.2 (2.9)

MI is only used for evaluation purposes to see if there will be mitotic cells enough for the analysis of chromosome aberrations. As pointed out earlier, it has been found that with an index below 10 there will normally be too few anaphase and telophase cells to score at the slide.

The *Allium* anaphase–telophase chromosome aberration assay was developed as a modification of the *Allium* test described by Fiskesjö (1985) to make a simpler and faster assay for detection of the genotoxicity of chemicals and environmental samples. Fortunately, the test system has been found useful for many different studies. Odeigah et al. (1997) used the *Allium* test to show the genotoxicity of wastewater from an oil field, and Monarca et al. (2000) investigated urban wastewater disinfected by different chemicals and showed a positive response in the *Allium* test when peracetic acid was used as a disinfectant. The *Allium* test also showed good results when aqueous extracts from lead-contaminated soils before and after remediation were examined for genotoxic effects (Chang et al., 1997). Further, in a soil study using the *Allium* test, Kovalchuk et al. (1998) found a strong, significant correlation of chromosome aberrations with ^{137}Cs activity in soils contaminated by the Chernobyl accident.

In conclusion, the *Allium cepa* anaphase–telophase chromosome aberration assay is useful for many types of environmental samples and can be recommended as a tool for monitoring the genotoxic effects and thereby contributing to environmental risk assessment means, which are becoming ever more important.

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ALLIUM ANAFAZINIŲ-TELOFAZINIŲ CHROMOSOMŲ ABERACIJŲ TYRIMO METODAS

S a n t r a u k a

Svogūno *Allium* anafazinių-telofazinių chromosomų aberacijų tyrimo metodas buvo sukurtas greitam cheminių medžiagų ir aplinkos pavyzdžių genotoksikologiniam įvertinimui. Prieš genotoksiškumo tyrimą atliekamas 96 val. trukmės šaknelių augimo inhibicijos testas. Chromosomų aberacijos nustatomos šakneles paveikus tiriamąja medžiaga 48 val. Klastogeniškumo rodikliai yra atsirandantys chromosomų tiltai ir fragmentai, o atsiliekančios ir pasimetusios chromosomos rodo aneugeninį poveikį. Metodas yra greitas bei patikimas ir gali būti taikomas nutekamiesiems vandenims, upės vandenims, užterštoms dirvoms ir sudėtingiems mišiniams tirti.

Raktažodžiai: *Allium cepa*, genotoksiškumas, chromosomų aberacijos, anafazė, telofazė