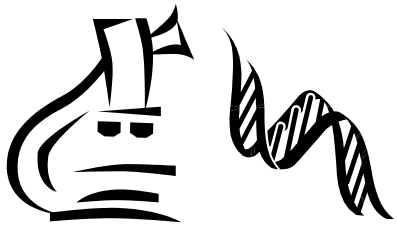
PRIVATE LABORATORY

OF PLANT CYTOGENETICS



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ALLIUM METAPHASE GENOTOXICITY ASSAY FOR THE TESTING OF DRINKING WATER, CHEMICALS, AND ENVIRONMENTAL SAMPLES

Test report

Evaluation of quality improvement of waterworks tap water treated in accordance with original technology TP in GLASS »i91422 sport«

Localities of the water samples: *Mariborska c. 12, SI – 3000 Celje, Slovenia*

Equally – treatment with GLASS »i91422 sport«

Cytogenetics research was performed betwen 9th and 15th April, 2013

Commissioner for:
i91422 ltd, 51A Anson Road, London

Domžale, 16th APRIL, 2013

1. SUMMARY

Allium assay is a test used for establishing genotoxicity of earth ecosystems - water, ground, air, and other media. Frequency of chromosomal aberrations (CA) is established in root tip cells of the test plant roots – common onion (Allium cepa L.) where evidence of potential genotoxic substances is provided. The test takes into account comprehensive influence and mutual action between genotoxic substances and genetic material (chromatin, chromosomes).

Onion tests (Allium tests) give unyielding evidence of the quality of water and unlike chemical tests show the comprehensive pollution effects, not only the presence of substances sought by methods employed by analytical chemistry.

2. INTRODUCTION

Allium test is a plant test that is important for conducting research on waters (drinking water, surface stagnant and flowing waters, communal waste waters, drainage waters of waste depots and meteoric waters). Its use was recommended as early as in the 1970's by the Royal Swedish Academy of Science (1973) and later by the GENE – TOX PROGRAM (Grant 1982). The advantage of this test in comparison with others is that it does not require preliminary processing of water samples. At the same time the Allium test is used for establishing general toxicity and genotoxicity. It also shows an excellent correlation with tests where research on fish and mammals in vivo is carried out (Fiskesjö 1985, Al-Sabti 1992, Firbas 2004; 2011). Results can be extrapolated with reliability to humans. The test is also useful for monitoring and supervising the burden of poisonous substances in the environment INVITTOX – PROTOCOL 8 (IP – 8 © September 1989). The International Programme on Plant Bioassays (IPPB) has acknowledged Allium test for biomonitoring and testing of environment polluters. Allium test has been standardised and validated in the framework of the above mentioned programmes.

Due to uncontrolled releases in the environment by industry, intensive farming and also tourism a number of chemical substances accumulate in the environment, esp. in water. In certain concentrations these substances have a mutagenic effect on organisms, which causes various degrees of genetic material damage and injuries (Firbas 1999), not to speak of industrial chemicals, metals, chemicals of pharmaceutical origin, hormone disruptors ad other pollutants. Even concentrations smaller than 0,1 µg (0,1 ppb – part per billion) of pesticide products have a partial inhibitory effect on the growth of test plant roots and are the cause of chromosomal and chromatid aberrations in cells (Firbas 2003; 2007).

3. MATERIAL AND METHODS

The test is run according to: Technical Methods Section 1993, 1994: INVITTOX – Protocol No.8, 1989; Fikesjö, 1985; Al-Sabti 1989; Nielsen 1994; Rank 2003; Firbas 2004, 2006, 2011; Kumar and Panneerselvam 2007; Ragunathan and Panneerselvam 2007.

Five bulbs of *Allium cepa* L. are used for each sample and both controls. All the samples and both controls are grown on a given medium for 72 hours. Results of general toxicity are indicated by the length of test plants roots. In the root tip cells chromosomal aberrations are assessed which indicate the genotoxicity level. Negative control is tap water filtered through

a two-stage filtration system R. O. reverse ossmosis). Positive control is 1 mg/L or 1 ppm methane-methyl- sulphamide – MMS 4016, SIGMA.
Cytogenetical research is carried out with research microscope Olympus – BX 41 Japan) with automatic photosystem PM 10 SP, at X400 and X1000 enlargement.

4. RESULTS AND DISCUSSION

Allium test was used in the investigation of the following water samples at the locations: *Mariborska c. 12, SI – 3000 Celje, Slovenia*

Results of general toxicity are given and the genotoxicity level is given in tables 1.

Integral parts of Allium test are the so called negative and positive control.
Negative control shows the degree of toxicity in unexposed onions and serves as control of the test efficiency.
Positive control is used with known material which normally induces a high degree of toxicity and is necessary for controlling the test response. In other words: the nearest the results of tested samples to negative control, the better the quality of water; or the other way, the farthest the values from the results of negative control and the nearest to positive control, the poorest the quality of water.

The application of Allium test enables us to give two kinds of results: general toxicity (indicated by root length of test plants) and genotoxicity level. The genotoxicity level (Al-Sabti 1989; Firbas 2004, 2006, 2010) is expressed as a percentage ratio between all metaphase cells and cells which present chromosomal aberrations.

4.1. General toxicity

ANALYSIS RESULTS OF GENERAL TOXICITY OF DRINKING WATER INDICATE THE ROOT LENGTH OF TEST PLANTS (Figure 1)

4.2 Genotoxicity leve

ANALYSIS RESULTS OF GENOTOXICITY LEVEL OF DRINKING WATER INDICATE CHROMOSOMAL ABERRATIONS IN ROOT TIP CELLS OF TEST PLANTS. INSPECTION OF UP TO 200 METAPHASE CELLS, AT A HIGH LEVEL OF GENOTOXICITY UP TO A MAXIMUM OF 100 CELLS (Figure 2 and 3)

Localities of the water samples: *Mariborska c. 121, SI – 3000 Celje, Slovenia*
Equally – treatment with original technology TP in GLASS »i91422 sport«

I. Waterworks water; Mariborska c. 121, SI – 3000 Celje,
II. Treated waterworks water with GLASS “i91422 sport”
III. Negative control (tap water filtered through R. O. – Reverse Osmossi)
IV. Positive control (1 mg/L or 1 ppm methane-methyl-sulphamide – MMS 4016, SIGMA)

GENERAL TOXICITY

Results of general toxicity are given in Table 1.

As regards general toxicity waterworks water samples (Sample I and II) vary minimally.
As regards average root length all samples of waterworks water have statistically longer roots than the roots of positive control samples (Sample IV).
The sample of treated waterworks water (Sample II) exhibits the lowest level of general toxicity (longest root length).

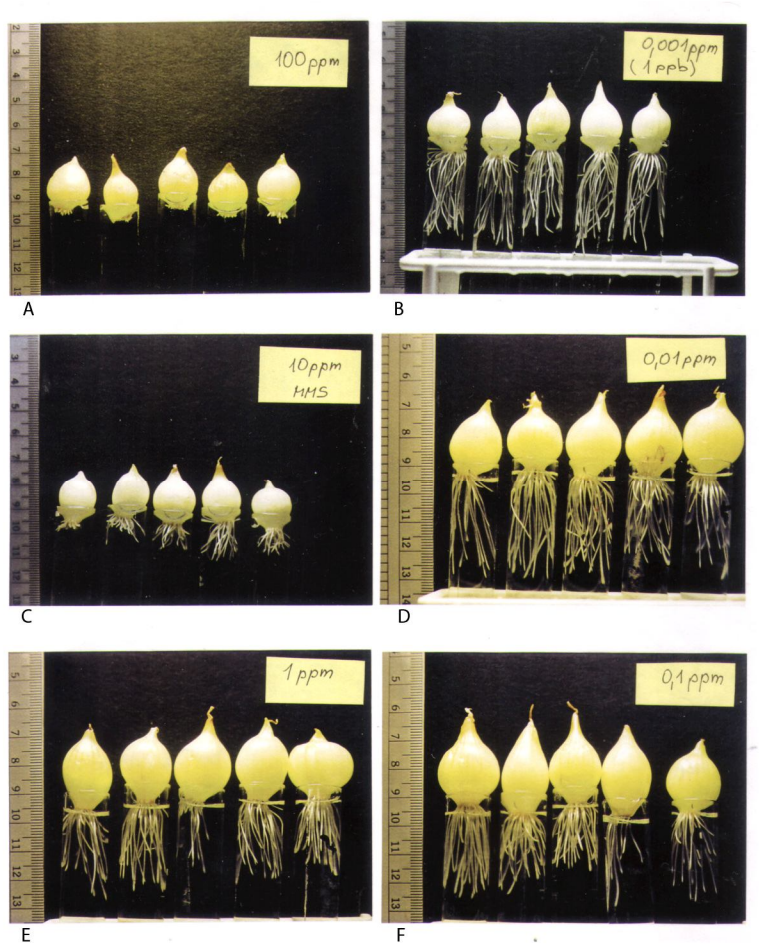

Fig. 1 – Examples of series of onions cultivated for 72 hour in different concentration MMS: 100 ppm (1a), 10 ppm (1c), 1 ppm (1e), 0,1 ppm (1f), 0,01 ppm (1d) and 0,001 ppm (1b).

GENOTOXICITY LEVEL

Results of cytological analysis (genotoxicity level) are given in Table 1.

The following chromosomal aberrations were observed: the most frequent are chromosome breaks in the primary constriction (centromere region); in a chromosomal set a maximum of two chromosomes is damaged.
In positive control samples single-strand and double-strand chromatide breaks were also observed as well as circular chromosomes.

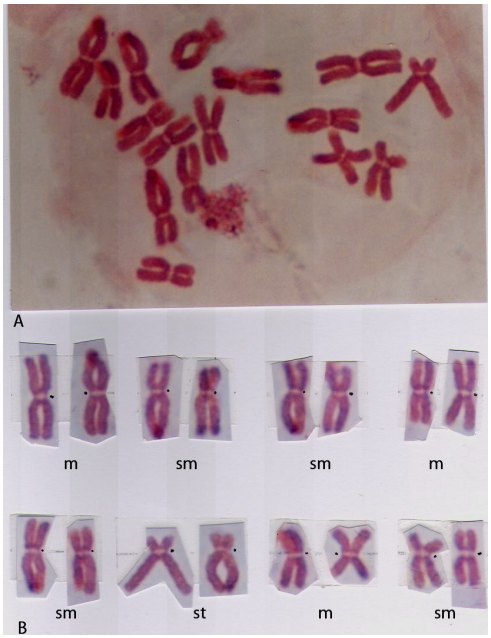

Fig. 2 – Diploid methaphase chromosome (2A) and its karyotype from the root cells of the onion (*Allium cepa* L.) containing 2n of 16 (2n =16) with 6 m, 8 sm and 2 st chromosomes (2B).

Table 1. Cytological effects of investigated samples and both controls – investigation of genotoxicity level and Average root length of test plant *Allium cepa* L. – investigation of general toxicity (water samples taken: 2013-04-09.)

Sample	Duration of sample plants cultivatio	Number of metapfase cells	Number of metaphase cells with chromosome aberations	Genotoxicity level (%)	Average root length (mm)
I	72	200	37	18,5*	33,5
II	72	200	21	10,5*	41,2
III	*72*	*200*	*5*	*2,5*	*42,0*
IV	*72*	*200*	*38*	*19,0*	*24,5*

Sample of waterworks tap water (Sample I) and sample of treated water (Sample II) are statistically different because the treatment reduces the genotoxicity level of water.

All the water samples are statistically different from positive control (Sample IV), where the results of genotoxicity level (Samples I and II) are lower than in positive control (Sample IV).

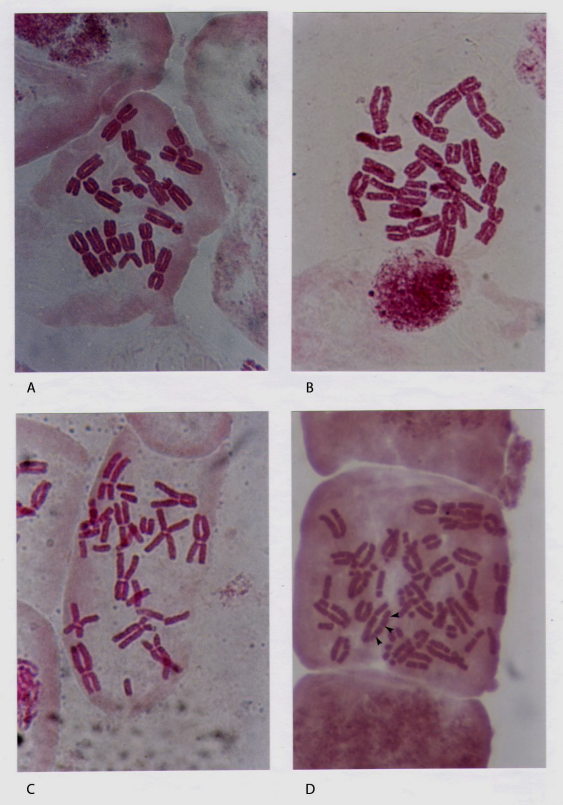

Fig. 3 – Different number chromosome damage in metaphase cells obtained from the meristeme root-type cells of onion (*Allium. cepa* L.): one damaged chromosome (3A), four damaged chromosome (3B), eight damaged chromosomes (3C), whole chromosome garniture is damaged (3D).

5.GENERAL CONCLUSIONS

1. Following the results of general toxicity and genotoxicity level between positive and negative control treated tap water (original GLASS "i91422" sport) has a lower genotoxicity level than ordinary tap water.
2. Treatment in accordance with original technology (original GLASS "i91422" sport) reduces the level of general toxicity as well as the level of genotoxicity ($p = 0,0325 < 0,05$ – Fisher's Exsact Test). The quality of water is improved.

6. RESEARCH GOALS

Introduction of genotoxicity research in environment protection policies is of great importance because it enables us to understand the impacts and consequences of genotoxic substances present in water. The goal of our research is to give an immediate and important contribution to preserving the health of the most precious life source – water. Due to our lack of knowledge and carelessness we have already polluted some water sources, therefore it is our obligation to correct our mistakes eagerly and wilfully. Time has come to take care also of posterity. We should be well aware that as regards genotoxicity there are no safe "maximum permissible concentrations" (MPC) which would ensure a good and reliable quality of water.

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DOMŽALE, 16th april, 2013

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