# Enhancement of micronuclei frequency in the *Tradescantia*/micronuclei test using a long recovery time

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The *Tradescantia*/micronuclei test (TRAD/MCN) is a well-validated test for monitoring environmental genotoxicants. These pollutants induce at the early meiotic stage of pollen mother cells chromosome fragments which become micronuclei at the tetrad stage. The standard test protocol requires some hours of exposure of the inflorescences and a *recovery time* of about 24 hours to reach the early tetrad stage. Since the recovery period represents a critical step of the TRAD/MCN, experiments were performed to establish its length in plants of clone # 4430 of the hybrid *T. hirsutiflora* × *T. subacaulis* which is widely used in environmental monitoring. The aim of the present research was to ascertain the exact duration of recovery time in order to improve the sensitivity of the TRAD/MCN test. First, studies were performed to select the flowers at the beginning of the meiosis, and then anthers were sampled and studied for a period of 48–86 hours. The complete meiosis in the plants examined required about 80 hours. Second, exposure to genotoxic substances followed by different recovery times was carried out to demonstrate that effectiveness of the TRAD/MCN test is closely related to the duration of the recovery time. The test was carried out by exposing inflorescences to known mutagens (sodium azide and maleic hydrazide) for six hours followed by different recovery times (24–72 hours). The results showed that the frequency of micronuclei in the pollen mother cells increased with the length of the recovery time.

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Many studies have shown that air, water and soil are frequently contaminated with mutagens and carcinogens, which can come into contact with humans and increase environmental carcinogenic hazards. Therefore, monitoring genotoxins in the environment has become an important objective of public health aimed at avoiding or minimizing direct and indirect human exposure to these toxic compounds. Plant tests have been widely used for detecting the genotoxicity of chemical substances and especially for in situ monitoring of environmental genotoxic pollutants in environmental media without subjecting samples to preliminary concentration, as also reported in a recent special issue of Mutation Research by the Co-ordinator of the International Program on Plant Bioassays sponsored by the United Nations Environment Programme (MA 1999). The *Tradescantia* micronuclei test (TRAD/MCN) is one of the most widely used and validated plant bioassays because it is quick, simple and inexpensive and can be widely adopted by many laboratories for screening chemicals and monitoring air, soil and water pollution. It was developed in 1976 to study the clastogenic effect of 1,2-dibromoethane (MA et al. 1973), and was then used to detect the effects of X-rays (MA 1979), well-known mutagens such as EMS, sodium azide, hydrazoic acid, cyclohexylamine and maleic hydrazide, many other chemical substances as well as common foods and drugs (MA et al. 1984) and numerous environmental clastogens (MA 1990; MA et al. 1982). Due to its sensitivity and versatility, the test has been used in the monitoring of external air (MA et al. 1996; MONARCA et al. 1999b), gas stove emissions (MONARCA et al. 1998b), soil (CABRERA and RODRIGUEZ 1999b; COTELLE et al. 1999; GICHNER and VELEMÍNSKÝ 1999), wastewater (GRANT et al. 1992; RUIZ et al. 1992; MONARCA et al. 1999a), and drinking water (MA et al. 1984; 1985; MONARCA et al. 1998a).

The test depends on the extreme sensitivity of the meiotic chromosomes of some species of *Tradescantia* to genotoxic substances. These substances break the DNA molecules and generate chromosomal fragments that appear as micronuclei in the cells of the pollen tetrads. The test consists of two steps: exposure and recovery time. During the first step, the flowers are exposed to toxic substances and then returned to normal environmental conditions, and kept in pure water or in nutrient solution. The second step, called recovery time, is the time it takes the meiocytes to pass from the initial part of prophase I

on to the tetrad phase. The duration of the recovery time is of crucial importance for the results of the test: the closer the recovery time coincides with the time required to complete meiosis, the more of the tetrads examined will be those deriving from the cells in prophase I at the time of contact with the toxic substance.

The aim of this research was to ascertain the meiotic cycle in the hybrid *T. hirsutiflora* × *T. subacaulis* to improve the sensitivity of the TRAD/MCN test. For this purpose flowers at the beginning of meiosis were identified on the basis of their size, then the mean duration of meiosis was determined in order to establish the length of recovery time. Finally, exposure to known genotoxic substances followed by different recovery times was carried out to demonstrate that the effectiveness of the TRAD/MCN test is closely related to the duration of the recovery time.

## MATERIALS AND METHODS

### *Duration of meiosis*

Single flowers of the hybrid *T. hirsutiflora* × *T. subacaulis* (clone # 4430) from young inflorescences were excised and measured under a stereomicroscope. The anthers of each flower were then extracted, squashed on a slide with some drops of acetocarmine and observed under the microscope.

Investigations to determine the duration of meiosis were performed by removing anthers one by one from the same flower at set time intervals of 10, 12 or 15 hours. A total of 20 flowers were used to determine the mean time it took meiocytes to pass from the initial prophase stage on to the tetrad stage.

### *Evaluation of the TRAD/MCN test with different recovery times*

The experiments were carried out using two known mutagenic substances: sodium azide ($NaN_3$, BDH) and maleic hydrazide (MH, SIGMA).

The first experiment was carried out with a solution of 0.2 mmoles/l of sodium azide and a solution of 0.2 mmoles/l of maleic hydrazide with 6 hours of exposure and five different recovery times: 24, 36, 48, 60 and 72 hours. These concentrations were chosen according to previous studies (MA et al. 1994). Tap water was used as the control.

A second experiment was carried out using different concentrations of mutagens solutions. Three solutions of different concentrations were prepared for each of these substances: 0.1, 0.2 and 0.4 mmoles/l for $NaN_3$ and for MH. Three assays with different recovery times were carried out for each solution. The exposure time was always 6 hours, as reported by other authors (HELMA et al. 1995; STEINKELLNER et al. 1998; YANG 1999). A 24-hour recovery time was used by some authors (CABRERA and RODRIGUEZ 1999a; CABRERA et al. 1999; GICHNER and VELEMÍNSKÝ 1999; MA 1983; MA et al. 1980; 1994; ZENG et al. 1999) and was compared with longer periods (72 hours).

The slides were prepared according to MA et al. (1994) by squashing the anthers of a single flower on a slide with drops of acetocarmine and placing on a cover slip. The slides were examined under the microscope to observe the meiotic phase. The frequency of micronuclei (MCN) was calculated for a total of 300 tetrads per slide and the results are expressed as a percentage (MCN/100 tetrads). Five slides were analysed for each exposure. Statistical differences

Table 1. *Tradescantia inflorescences (T. hirsutiflora × T. subacaulis, clone # 4430) tested with maleic hydrazide and sodium azide (0.2 mmoles/l) with different recovery times and doses*

| Treatment | Exposure (hr) | Recovery time (hr) | MCN/100 tetrads (Mean ± SD) | |
|---|---|---|---|---|
| | | | Treatment | Control |
| Maleic hydrazide (0.2 mmoles/l) | | | | |
| | 6 | 24 | 7.12 ± 1.90 | 6.50 ± 1.14 |
| | 6 | 36 | 13.68 ± 2.53** | 7.28 ± 1.61 |
| | 6 | 48 | 15.40 ± 2.76* | 9.02 ± 3.84 |
| | 6 | 60 | 20.96 ± 8.57** | 7.04 ± 0.96 |
| | 6 | 72 | 26.84 ± 4.33** | 8.89 ± 1.60 |
| Sodium azide (0.2 mmoles/l) | | | | |
| | 6 | 24 | 3.28 ± 2.10 | 1.36 ± 0.55 |
| | 6 | 36 | 11.60 ± 3.36** | 2.54 ± 1.34 |
| | 6 | 48 | 7.62 ± 1.84** | 1.40 ± 0.83 |
| | 6 | 60 | 17.04 ± 2.79** | 2.72 ± 1.20 |
| | 6 | 72 | 35.18 ± 9.13** | 2.64 ± 0.81 |

* Statistically significant according to Dunnett's *t*-test ($p < 0.05$)
** Statistically significant according to Dunnett's *t*-test ($p < 0.01$)

Table 2. *Inflorescences tested with maleic hydrazide with different recovery times and doses*

| Maleic hydrazide (mmoles/l) | Exposure (hr) | Recovery time (hr) | MCN/100 tetrads (Mean ± SD) |
|---|---|---|---|
| 0 | 6 | 24 | 7.4 ± 1.9 |
| 0.1 | 6 | 24 | 10.5 ± 1.3 |
| 0.2 | 6 | 24 | 12.2 ± 2.8** |
| 0.4 | 6 | 24 | 16.3 ± 2.1** |
| 0 | 6 | 72 | 7.5 ± 0.6 |
| 0.1 | 6 | 72 | 14.9 ± 2.2** |
| 0.2 | 6 | 72 | 13.4 ± 2.8** |
| 0.4 | 6 | 72 | 19.0 ± 3.5** |

* Statistically significant according to Dunnett's *t*-test ($p < 0.05$)
** Statistically significant according to Dunnett's *t*-test ($p < 0.01$)

between the scores obtained in the experiments and the controls were tested using Dunnett's test.

## RESULTS AND DISCUSSION

The tests carried out have shown that in clone # 4430 the meiotic cycle takes place in 84–86 hours. This comes from the observation that meiocytes take approximately 60 hours to pass from the early stage, called prophase I, on to metaphase I and anaphase I, and then about 20 hours to reach the tetrad stage.

These findings are similar to those obtained by TAYLOR (1950) for *Tradescantia paludosa*.

It has been already pointed out in the introduction that the reliability of the *Tradescantia*-micronuclei test is closely linked to the probability of finding micronuclei in the same cells damaged by the mutagens. It is therefore clear that the total duration of the recovery time and exposure must correspond as far as possible to the time for mejosis. According to these data, the widely used recovery time of 24 hours, seems to be in contradiction with the aims of the recovery time. Such a thesis has been confirmed by testing the influence of different recovery times on the results of the micronuclei test in inflorescences treated with known mutagens (sodium azide and maleic hydrazide).

Table 3. *Inflorescences tested with sodium azide with different recovery times and doses*

| Sodium azide (mmoles/l) | Exposure (hr) | Recovery time (hr) | MCN/l00 tetrads (Mean ± SD) |
|---|---|---|---|
| 0 | 6 | 24 | 7.4 ± 1.9 |
| 0.1 | 6 | 24 | 7.5 ± 2.5 |
| 0.2 | 6 | 24 | 9.2 ± 3.2 |
| 0.4 | 6 | 24 | 12.0 ± 3.9 |
| 0 | 6 | 72 | 9.0 ± 1.4 |
| 0.1 | 6 | 72 | 16.0 ± 3.9* |
| 0.2 | 6 | 72 | 19.9 ± 4.4** |
| 0.4 | 6 | 72 | 23.9 ± 3.7** |

* Statistically significant according to Dunnett's *t*-test ($p < 0.05$)
** Statistically significant according to Dunnett's *t*-test ($p < 0.01$)

The results obtained with 6 hours of exposure and different recovery times (24, 36, 48, 60 and 72 hours) to 0.2 mmoles/l of maleic hydrazide solution and 0.2 mmoles/l of sodium azide solution are shown in Table 1. A longer recovery time seems to favour MCN formation, therefore MCN was shown to increase with recovery times longer than 24 hours, where statistical differences were obtained in comparison with the negative control. Since the two substances were tested in different days, negative controls gave different micronuclei values due to the variability of the growing conditions.

A recovery time of 72 hours for inflorescences treated with maleic hydrazide was shown to cause a statistically significant increase in micronuclei at all the doses tested, whereas a 24-hour recovery time gave negative results at the lowest dose (Table 2).

Sodium azide tested with a recovery time of 24 hours gave consistently negative results, whereas a recovery time of 72 hours led to a significant increase in micronuclei at all the tested doses (Table 3).

The TRAD/MCN test is a very efficient, simple and inexpensive system for use with a variety of physical, chemical and environmental agents. Exposure to genotoxic substances followed by different recovery times showed that the effectiveness of the TRAD/MCN test is closely related to the duration of the recovery time. On the other hand, the duration of the meiotic cycle can be delayed for hours or even days by certain treatments with toxic substances or when an overdose of an agent is used (MA 1983). Therefore, the problem connected with this meiotic delay can be overcome by means of a longer recovery time to enable the chromosomes damaged in the early prophase to reach the early tetrad stage.

The need to increase the sensitivity of the TRAD/MCN is evident when in situ monitoring of environmental mutagens is carried out in order to detect low levels of genotoxicants.

In conclusion, by using a simple modification to the test, namely a longer recovery time, we found increased sensitivity and the possibility of revealing lower levels of environmental genotoxins. We suggest testing environmental complex mixtures and matrices with the standard recovery time (24 hours) and, when the results become negative, repeating the experiments with a longer recovery time.

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