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# Soil quality — Assessment of genotoxic effects on higher plants — *Vicia faba* micronucleus test

*Qualité du sol — Évaluation des effets génotoxiques sur les végétaux supérieurs — Essai des micronoyaux sur* Vicia faba

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# Foreword

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The committee responsible for this document is ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

# Introduction

In the field of assessment of the quality of soils and soil materials, it appears necessary to determine *in vivo* their genotoxic potential which may be induced by pollution or by a decontamination process. Indeed, genotoxic agents have the ability to damage the genome of living organisms or to interfere with its functioning, but they are not always detected by chemical analysis or classical ecotoxicological tests. Actually, genotoxic effects are often observed at sublethal concentrations, where no toxic effect (e.g. survival or growth) can be observed in the short term but some long term effects may be feared in living organisms. Moreover, higher plants, like *Vicia faba* (broad bean) are ecologically relevant to assess soils and soil materials quality.

# Soil quality — Assessment of genotoxic effects on higher plants — *Vicia faba* micronucleus test

## 1 Scope

The purpose of this International Standard is to describe a method for assessing genotoxic effects (chromosome breakage or dysfunction of the mitotic spindle) of soils or soil materials on the secondary roots of a higher plant: *Vicia faba* (broad bean). This method allows the assessment of genotoxicity (toxicity for genetic material) of soils and soil materials like compost, sludge, waste, fertilizing matters, etc. Two ways of exposure can be considered: a direct exposure of plants to the soil (or soil material) which is relevant for the real genotoxic potential and an exposure of plants to the water extract of the soil (or soil material). This last way of exposure to a leachate or an eluate allows the detection of the mutagens which are not adsorbed to soils and which may be transferred to aquatic compartments. Moreover, this test may be used to evaluate genotoxic effects of chemical substances and to waters, effluents, etc.

## 2 Normative reference

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11260, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

ISO 11269-2:2012, *Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants*

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

ISO/TS 21268-1, *Soil quality — Leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil materials — Part 1: Batch test using a liquid to solid ratio of 2 l/kg dry matter*

ISO/TS 21268-2, *Soil quality — Leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil materials — Part 2: Batch test using a liquid to solid ratio of 10 l/kg dry matter*

EN 14735, *Characterization of waste — Preparation of waste samples for ecotoxicity tests*

## 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**control soil**
uncontaminated substrate used as control and dilution medium for preparing dilution series with test soils or test materials

EXAMPLE compost, sludge, waste, chemicals

**3.2**
**mitotic index**
number of cells in division per 1 000 cells observed when all of the stages of the mitosis are taken into account, from the prophase (when the chromosomes begin to condense) up to the telophase (when the chromatin of the two nuclei formed at each pole of the cell finishes decondensing).

**3.3**
**test mixture**
mixture of test material (soil, compost, sludge, waste or chemical) with control soil

## 4 Principle

This genotoxicity test is based on the detection of micronuclei in the cells of the secondary root tips of *Vicia faba* (broad bean). The micronuclei, visible in the cytoplasm of the cells, result from a chromosome break (effect of clastogenic substances) or from a dysfunction of the mitotic spindle (effect of aneugens).

In both cases, the fragments of chromosomes or the entire chromosomes cannot migrate to one of the poles of the spindle at the time of the anaphase of the mitotic division and therefore form one (or more) micronucleus.

The micronucleus frequency is determined in the control root cells and in those which have been exposed to the soil (or soil material) or the water extract of the soil being tested. A statistical test then enables to determine the significativeness of data.

## 5 Plants, test equipment and reagents

### 5.1 Equipment

The exposure of the plants to the soils and soil materials under test is performed in plastic pots (diameter: 9 cm, height: 10 cm).

Exposure to water extract of soils is carried out in glass containers (e.g. glass beaker of capacity 200 ml).

A microscope equipped with an objective with x 400 magnification is required for studying the microscopic effects of the cells.

### 5.2 Test organism

The plant selected for its high sensitivity to micropollutants and for its ease of obtention is *Vicia faba* (broad bean), Aguadulce, with a very long pod. This higher plant forms part of the family of pulses and of the dicotyledoneae class.

Seeds coated with insecticides and/or fungicides should not be used.

### 5.3 Reference substance

Maleic hydrazide is recommended as a reference substance. The positive control is carried out at the concentration of $10^{-5}$ M, 1,12 mg/kg and 1,12 mg/l for solid-phase and liquid-phase exposures respectively.

The preparation of this photodegradable substance as well as the exposure of the plant organisms to the solution shall be carried out in the dark.

## 5.4 Reagents

### 5.4.1 Carnoy's solution

Carnoy's solution is composed of glacial acetic acid and of ethanol in respective volume proportions of 25 % and 75 % and shall be prepared extemporaneously.

### 5.4.2 Hydrolysis solution

A solution of HCl with concentration 1 mol/l enables to conduct the hydrolysis of the roots.

### 5.4.3 Staining solution

The staining solution used for specifically highlighting the DNA is 1 % orcein diluted in 45 % acetic acid. This mixture is brought to the boil during 10 min, then filtered after cooling down. When it is used, it is important to filter the staining solution after each use in order to prevent the forming of orcein crystals which could be confused with micronuclei during the microscopic examination of the cells.

NOTE Other specific DNA staining solution can be used.

### 5.4.4 Hoagland's medium

Nutritive medium of which the composition is given in Annex A.

### 5.4.5 Intermediate solvent

Dimethyl sulfoxide (DMSO), at a maximal concentration of 1 %.

NOTE Any other appropriate water-miscible solvent whose genotoxic innocuousness has been previously established may be used.

# 6 Protocols

## 6.1 Preparation of the soil to be tested

### 6.1.1 Chemical substances

Chemical substances may be tested: their preparation is explained in Annex B.

### 6.1.2 Soils and soil materials

Whatever the soil to be tested (sampled from a contaminated site or from a remediated soil, or other soil materials like compost, sludge, waste, fertilizing matters, etc.), it should have pH values after sieving within a range that is not toxic to *Vicia faba*. Soils under test should be sieved by 4 mm mesh and thoroughly mixed and should be stored as shortly as possible, in the dark at 4 °C ± 2 °C in accordance with ISO 10381-6 using containers that minimise losses of soil contaminants by volatilisation and sorption to the container walls. Soil pH should not be corrected.

For each soil to be tested, the following characteristics should be determined:

— Soil texture classification,

— pH in accordance with ISO 10390,

— Water content in accordance with ISO 11465,

— Water holding capacity according to Annex B of ISO 11269-2:2012,

— Cationic exchange capacity in accordance with ISO 11260,

— Organic matter content in accordance with ISO 10694.

The soil mixtures are placed in plastic pots with a moisture content of 70 % of water-holding capacity.

### 6.1.3 Control soil

Either reference or standard natural soils can be used as control soil, e.g. LUFA soils[1)] previously air dried at room temperature, sieved between 2 mm and 5 mm, with clay (< 2µm) content < 25 % , silt (2 µm -50 µm) content < 45 % , soil organic matter content between 1,5 % and 5 % , $pH_{water}$ between 5 and 8 .

When comparing soils of known and unknown quality, the control soil and soil under test should be of the same textural class, and be as similar as practicable in all respects other than the presence of the chemical or contaminant being investigated. Indeed, significant differences in soil characteristics other than the presence of contaminants may lead to differences in plant cell division, so in micronucleus frequency and may induce false positive test results.

NOTE Although mitotic index is not modified by pH between 4 and 9, it is recommended to use a control soil with a $pH_{water}$ between 5 and 8 for a better genotoxicity assessment of chemicals.

### 6.1.4 Water extracts of soil

Water extracts of soils or soil materials are prepared, as rapidly as possible after receipt of the sample at the laboratory, with a leaching test according to one of the protocols described in ISO/TS 21268-1 or ISO/TS 21268-2 or EN 14735. However, the eluates obtained shall not be filtered but can be decanted during 2 h. In this case, the supernatant phase is sampled and stored in the dark at a temperature of 4 °C ± 3 °C up until the test is carried out which shall take place at the maximum 24 h after the leaching stage. The Hoagland's medium is used for the negative control and to prepare the dilutions of the water extract.

## 6.2 Preparation of the seeds

Seeds (approximately three times higher than the required number) are selected from the stock of seeds stored at 4 °C in the dark. Then a germination step is necessary to obtain secondary roots: the seeds are cleaned with demineralised water and immersed during a period between 6 h and 24 h at ambient temperature in demineralised water in order to hydrate them. The seed coats are then removed and the seeds are left to germinate vertically at 24 °C ± 1 °C in continually humidified cotton (not having undergone any chlorinated treatment) in the dark.

NOTE Other germination material may be used: vermiculite, peat, etc.

After about three days, only those seeds whose primary root length is between 3 cm and 5 cm are selected. Their tip (around 5 mm) is then cut off in order to interrupt the growth of this main root and to stimulate that of the secondary roots.

For solid phase exposure, the primary rooted seeds are directly placed in soils for beginning the exposure of secondary roots.

For liquid phase exposure, the seeds are then placed, so as to immerse only the root, over a container containing some nutritive medium (Hoagland's medium (5.4.4)) at a temperature of 24 °C ± 1 °C in order to induce the secondary roots sprouting. This previously oxygenated medium is renewed every 24h. The secondary roots of the seeds reach a length of 1 cm to 2 cm after a period of four days; the seeds bearing these secondary roots are then used for the purpose of the test.

This germination step of the *Vicia faba* seeds, necessary in both ways of exposure, can be started four days and eight days respectively for solid-phase and liquid-phase exposure before beginning the test.

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1) LUFA soils are an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

## 6.3 Conducting of the test

### 6.3.1 Soils and soil materials

The dilutions of the test mixture are chosen within a geometric series with a factor not exceeding two and shall cover a large range of concentrations (e.g. from 0,01 % to 100 % ). These mixtures are prepared by diluting the soil with a reference soil.

Each test shall include a negative control without any test sample and a positive control (see 5.3).

The direct exposure of the plant organisms to the different concentrations of the soil is performed by placing the germinated seeds (at least three per dilution) in a plastic pot containing 200 g of the tested soil and/or mixtures (see Figure 1) throughout the exposure time between three and five days, according to obtain at least ten roots of 1 cm length.

**Figure 1 — Method of direct exposure of the *Vicia faba* seeds**

### 6.3.2 Water extracts of soil

The concentrations of the sample under test are chosen within a geometric series with a factor not exceeding two and shall cover a large range of concentrations (e.g. from 0,01 % to 100 % for matrices). This range of concentrations is prepared by diluting the sample with the previously oxygenated Hoagland's medium (see Annex A).

Each test shall include a negative control without any test sample and a positive control (see 5.3).

At the time of the test, the different solutions to be tested are extemporaneously brought up to a temperature of 24 °C ± 1 °C and well homogenised before exposure of plant organisms. This is carried out by placing the germinated seeds (at least three per concentration) in a glass container having a sufficient diameter in order to prevent, as far as possible, contact between the root tips and the container wall throughout the exposure time. The roots are immersed in a minimum volume of 200 ml of test solution (see Figure 2).

**Figure 2 — Method of aqueous exposure of the *Vicia faba* seeds**

The exposure time shall be at least 30 h, which corresponds to the approximate duration of the cell cycle. However the optimal exposure time recommended to detect genotoxic effects is 48 h to be sure that the cell cycle is ended and for a better practicability.

## 6.4 Test environment

The tests are performed in a climatic chamber (with an intensity of at least 5 000 lx and 16/8 photoperiod) at a temperature of 24 °C ± 1 °C. The liquid-phase test can also be carried out in darkness if necessary (e.g. maleic hydrazide).

## 6.5 Cell preparation

At the end of the exposure period, the roots are simply removed from the water extract, or carefully extracted from the soil. Then they are cleaned with deionised water and the last two centimeters of the secondary roots (ten or so roots per seed, chosen at random) are sampled and placed at 4 °C for a minimum duration of one night in Carnoy's solution. These root tips can then be stored on a long term basis in 70 % ethanol for a deferred observation or else can be hydrolysed in the case of an immediate observation.

The root tips are then placed in distilled water for 10 min, hydrolysed in the hydrolysis solution at 60 °C for 6 min and retransferred into distilled water for a few minutes.

For root cell observation, place a root tip on a slide after wiping it cautiously. Remove the first millimeter corresponding to the root cap and the meristematic region and, with the help of a scalpel, retain only the second millimeter which is made up of the subsequent generation of cells obtained after mitosis. Micronuclei scoring shall be done in this particular cells region (see Figure 3). All these steps may be done on a black background to see the different cell regions.

The staining of the DNA is carried out by crushing the root tips after addition of a drop of orcein; the coverslip can then be placed in position and squeezed to obtain a single layer of cells.

It is recommended to carry out at least two cell spreadings (obtained with two different roots) per seed.

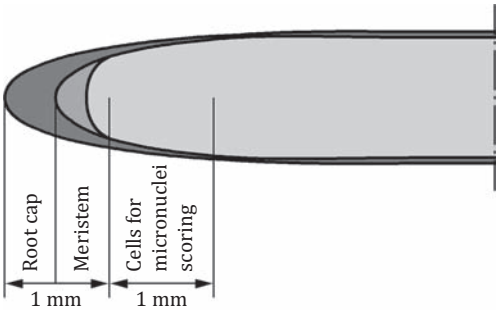


**Figure 3 — Longitudinal section of *Vicia faba* root showing the root cap, the meristem and cells region to be selected for micronuclei scoring**

It is preferable to perform the scoring under blindfold conditions prior to their examination so that the person conducting the test is not influenced when counting the micronuclei (see Figure 4). The micronuclei observed in cells in division shall not be taken into account when determining micronucleus frequency. The results are expressed in number of micronuclei per 1 000 cells in interphase.

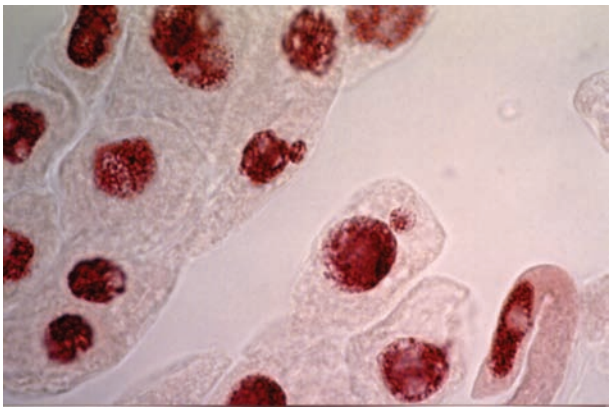

**Figure 4 — Micronuclei in the cells of the root tips of *Vicia faba* (x 400)**

During the micronuclei scoring, it is highly recommended to verify the proper progress of the cell division (an essential condition for the micronuclei formation) by determining the proportion of cells in mitotic division (commonly called mitotic index). A genotoxic effect can be masked by a cytotoxic one (toxic with respect to the cell functioning) which would induce an underestimation of the genotoxic potential of the sample under test. The results are expressed in number of cells in division per 1 000 cells observed. All the stages of the mitosis are taken into account, from the prophase (when the chromosomes begin to condense) up to the telophase (when the chromatin of the two nuclei formed at each pole of the cell finishes decondensing).

The microscopic examination of the slides is carried out under a magnification of 400. A minimum number of two slides is prepared for each of the three replicates for each concentration. Considering that 1 000 cells per slide are observed, the averages and standard deviations are therefore calculated on a minimum of 6 000 cells for each concentration.

# 7 Assessment of the results

## 7.1 Presentation of the data

The results of the negative and positive controls and of the concentrations under test are expressed in terms of average number of micronuclei per 1 000 cells observed.

## 7.2 Statistical analysis

The use of a non-parametric method (e.g. the Kruskal-Wallis test followed by Dunn's multiple comparison test) is recommended in order to highlight the significant differences between the control and test concentrations.

## 7.3 Interpretation of the results

### 7.3.1 Positive test

The test is considered as positive if a statistically significant result with respect to the negative control is detected for at least one of the test concentration.

### 7.3.2 Negative test

The test is considered as negative if, with respect to the negative control, no statistically significant positive response is observed for the tested concentrations. It is noteworthy that the absence of micronuclei in root cells may be due to a dysfunction of mitosis: in that case, DNA breakages are not excluded from the nucleus and do not form any micronucleus. Consequently, it is important to check if the mitotic index (number of cells in division) of each spreading is greater than 20 for 1000 cells, otherwise results of micronucleus frequency shall not be taken into account.

# 8 Validity criteria

The test is considered as valid if a positive response is obtained with the reference substance.

For each concentration, the micronucleus frequency is reliable if the mean mitotic index is greater than 20 cells in division for 1 000 observed cells.

# 9 Test report

The test report shall include the following information:

a) a reference to this International Standard;

b) a full description of the experimental methodology and procedures;

c) the origin of the broad bean seeds;

d) the test environment (temperature, photoperiod, lighting, etc.);

e) the characteristics of test soil (if appropriate);

f) the characteristics of test material: compost, sludge, waste (if appropriate);

g) the method of preparation of water extract of soils (if appropriate);

h) the characteristics of control soil;

i) the exposure time;

j) the observations of acute toxicity made during the course of the test (visible damage of the organisms: blackening of the roots, necrosis of certain parts of the plants, etc.);

k) the results as indicated in Clause 7, specifying the statistical methods used.

# Annex A
(informative)

# Composition of Hoagland's medium

See Table A.1.

**Table A.1 — Composition of Hoagland's medium**

| Products | Stock solution | Quantity of stock solution per 1 l of medium | Final concentration |
|---|---|---|---|
| $KNO_3$ | 50,5 g/l | 10 ml | 5 mmol/l |
| $Ca(NO_3)_24H_2O$ | 118,0 g/l | 10 ml | 5 mmol/l |
| $MgSO_4K,7H_2O$ | 123,2 g/l | 10 ml | 5 mmol/l |
| $KH_2PO_4$ | 13,6 g/l | 10 ml | 1 mmol/l |
| Iron tartrate | 500 mg/l | 10 ml | 9 $\mu$mol/l |

The stock solutions of Hoagland's medium can be stored for three months at 4 °C. It is recommended to prepare the Hoagland's medium at the time of use and to aerate the medium by bubbling through.

# Annex B
(informative)

# Testing chemicals added to soils

The stock solution of the chemical substance to be experimented is prepared by dissolving a known quantity of substance in a defined volume of deionised water. It shall be prepared at the time of use.

NOTE If it is shown analytically that the substance is stable in the dark and at 4 °C, it may be prepared in advance and stored under these conditions.

In aqueous exposure, the test solutions are prepared just prior to use by dissolving the stock solution in the dilution medium (Hoagland's medium, see Annex A) in order to obtain the necessary concentrations. In direct exposure, the different solutions are used to spike the reference soil.

In the case of barely water-soluble substances or substances that are insoluble in water, an intermediate water-miscible solvent (see (5.4.5) can be used. In this case, the concentration of the solvent in each container shall be constant and shall not exceed 100 µl/l. The dilution medium is shaken at the time of introduction of the intermediate solution, which generally leads to the forming of a microsuspension. If a precipitate appears, the test cannot be carried out. If the use of an intermediate solvent cannot be avoided, a negative control containing the same concentration of solvent shall be included in the test.

# Annex C
(informative)

# Results of the interlaboratory test conducted within the framework of NF T 90-327

## C.1 Results with $CdCl_2$

See Tables C.1 and C.2.

**Table C.1 — Proportion of micronuclei (number of micronuclei/1 000 cells) according to the concentration of $CdCl_2$**

| Laboratory | Negative control | | $7,5.10^{-8}$ mol/l | | $10^{-7}$ mol/l | | $2,5.10^{-7}$ mol/l | |
|---|---|---|---|---|---|---|---|---|
| | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 |
| France A | 3,83 ± 1,33 | 4,67 ± 1,75 | 17,83 ± 4,66[a] | 11,50 ± 3,27[a] | 30,00 ± 5,29[a] | 16,17 ± 3,97[a] | 39,33 ± 10,01[a] | 25,67 ± 5,05[a] |
| France B | 0,33 ± 0,52 | 0,17 ± 0,41 | 7,83 ± 3,25[a] | 5,33 ± 2,58[a] | 15,17 ± 4,02[a] | 11,33 ± 1,75[a] | 8,83 ± 6,62[a] | 5,83 ± 3,25[a] |
| France C | 0,33 ± 0,52 | 0,17 ± 0,41 | 12,50 ± 2,43[a] | 14,67 ± 2,66[a] | 16,83 ± 3,43[a] | 15,83 ± 2,93[a] | 20,17 ± 3,76[a] | 27,17 ± 3,19[a] |
| [a] Statistically significant difference from negative control (Kruskal Wallis test, $p$<0,05). | | | | | | | | |

**Table C.2 — Mitotic index (number of cells in division/1 000 cells) according to the concentration of $CdCl_2$**

| Laboratory | Negative control | | $7,5.10^{-8}$ mol/l | | $10^{-7}$ mol/l | | $2,5.10^{-7}$ mol/l | |
|---|---|---|---|---|---|---|---|---|
| | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 |
| France A | 51,17 ± 7,83 | 61,33 ± 14,78 | 54,50 ± 8,73 | 53,83 ± 11,36 | 79,50 ± 11,04 | 55,83 ± 15,22 | 70,33 ± 19,47 | 59,00 ± 14,18 |
| France B | 29,83 ± 7,94 | 24,00 ± 2,68 | 27,83 ± 8,13 | 22,83 ± 6,68 | 30,00 ± 12,30 | 24,67 ± 5,68 | 15,33 ± 5,20 | 10,33 ± 4,50 |
| France C | 36,83 ± 5,56 | 43,33 ± 4,37 | 38,50 ± 3,27 | 42,17 ± 4,53 | 34,50 ± 3,73 | 42,33 ± 4,18 | 38,33 ± 4,97 | 39,67 ± 6,47 |

## C.2 Results with maleic hydrazide

See Tables C.3 and C.4.

**Table C.3 — Proportion of micronuclei (number of micronuclei/1 000 cells) according to the concentration of maleic hydrazide**

| Laboratory | Negative control | | $5.10^{-6}$ mol/l | | $10^{-5}$ mol/l | | $2.10^{-5}$ mol/l | |
|---|---|---|---|---|---|---|---|---|
| | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 |
| France A | 5,33 ± 1,21 | 1,33 ± 1,97 | 10,00 ± 1,41[a] | 12,67 ± 5,20[a] | 39,17 ± 15,52[a] | 20,33 ± 3,72[a] | 10,33 ± 4,93[a] | 12,50 ± 3,56[a] |
| France B | 0,33 ± 0,52 | | 17,33 ± 8,91[a] | | 19,33 ± 5,28[a] | | 9,17 ± 6,31[a] | |
| France C | 0,50 ± 0,84 | 0,67 ± 0,82 | 13,67 ± 2,66[a] | 7,50 ± 1,22[a] | 57,83 ± 20,60[a] | 62,50 ± 13,58[a] | 9,33 ± 3,61[a] | 13,33 ± 3,56[a] |
| France D | 0,50 ± 0,84 | 0,50 ± 0,84 | 38,67 ± 20,25[a] | 32,25 ± 8,02[a] | 73,67 ± 27,53[a] | 67,00 ± 27,53[a] | 48,50 ± 24,3[a] | 44,33 ± 9,22[a] |
| France E | 0,33 ± 0,52 | 0,50 ± 0,84 | 13,50 ± 2,74[a] | 10,83 ± 2,93[a] | 44,17 ± 6,18[a] | 40,67 ± 8,14[a] | 11,17 ± 2,23[a] | 12,83 ± 3,13[a] |

[a] Statistically significant difference from negative control (Kruskal Wallis test, $p<0,05$).

**Table C.4 — Mitotic index (number of cells in division/1 000 cells) according to the concentration of maleic hydrazide**

| Laboratory | Negative control | | $5.10^{-6}$ mol/l | | $10^{-5}$ mol/l | | $2.10^{-5}$ mol/l | |
|---|---|---|---|---|---|---|---|---|
| | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 |
| France A | 40,00 ± 5,97 | 29,00 ± 10,94 | 43,83 ± 9,30 | 23,17 ± 8,95 | 40,67 ± 8,55 | 20,67 ± 7,26 | 19,67 ± 7,63 | 25,00 ± 12,03 |
| France B | 79,50 ± 6,47 | | 50,50 ± 5,47 | | 45,50 ± 10,93 | | 22,00 ± 6,07 | |
| France C | 23,50 ± 2,51 | 22,00 ± 2,61 | 22,50 ± 1,87 | 20,50 ± 2,26 | 18,00 ± 3,35 | 16,67 ± 2,94 | 7,33 ± 5,99 | 3,17 ± 2,86 |
| France D | 40,83 ± 20,93 | 52,33 ± 7,23 | 42,00 ± 12,46 | 24,75 ± 11,18 | 33,50 ± 8,31 | 25,50 ± 14,43 | 17,83 ± 13,61 | 17,83 ± 9,58 |
| France E | 47,67 ± 9,00 | 46,33 ± 8,41 | 48,33 ± 11,43 | 44,83 ± 8,13 | 44,83 ± 9,06 | 47,00 ± 9,84 | 12,50 ± 3,39 | 14,33 ± 4,41 |

# Annex D
(informative)

# Results of the interlaboratory test conducted on the reference substance and an industrial contaminated soil

The aim of this ring test, organized by LIEBE-CNRS 7146 (University of Metz, France) was to check if the *Vicia*-micronucleus test could be used in different countries, so each participant used its own reference soil and bought its own seeds. The most important variability sources were chosen to assess the robustness of the test rather than the reproducibility. Maleic hydrazide, recommended as a reference substance, was tested by the two ways of exposure: respectively 10 µmol/kg (1,12 mg/kg) and 10 µmol/l (1,12 mg/l) for solid-phase and liquid-phase exposures. Each participant had to prepare the spiked soil mixing its own reference soil and the maleic hydrazide.

## D.1 Reference substance results

See Tables D.1 to D.4.

**Table D.1 — Micronucleus frequency (number of micronuclei/1 000 cells) for the maleic hydrazide (1,12 mg/kg) in solid phase**

| | negative control | positive control |
|---|---|---|
| France L1 | 0,2 ± 0,4 | 8,5 ± 2,4[a] |
| France L2 | 0,0 ± 0,0 | 32,0 ± 10,6[a] |
| France L3 | 0,2 ± 0,4 | 73,7 ± 18,9[a] |
| France L4 | 0,0 ± 0,0 | 5,8 ± 1,5[a] |
| France L5 | 1,8 ± 0,6 | 12,4 ± 1,2[a] |
| Italy | 0,3 ± 0,5 | 85,7 ± 17,2[a] |
| Brazil | 0,2 ± 0,4 | 8,5 ± 3,3[a] |

[a] Statistically significant difference from negative control (Mann-Whitney U test, $p<0,05$).

Micronucleus frequency in root cells of negative controls is normally low

Micronucleus frequency of positive controls is significantly different from the control

**Table D.2 — Mitotic index (number of cells in division/1 000 cells) for the maleic hydrazide (1,12 mg/kg) in solid phase**

| | negative control | positive control |
|---|---|---|
| France L1 | 96,3 ± 8,7 | 70,2 ± 7,1 |
| France L2 | 93,9 ± 15,1 | 148,3 ± 24,8 |
| France L3 | 112,5 ± 20,5 | 74,3 ± 15,3 |
| France L4 | 52,8 ± 9,2 | 29,2 ± 8,2 |
| France L5 | 87,8 ± 22,2 | 78,7±9,3 |
| Italy | 143,0 ± 5,5 | 111,8 ± 9,7 |
| Brazil | 36,8 ± 7,1 | 24,2 ± 5,2 |

**Table D.3 — Micronucleus frequency (number of micronuclei/1 000 cells) for the maleic hydrazide (1,12 mg/l) in liquid phase**

| | negative control | positive control |
|---|---|---|
| France L1 | 0,2 ± 0,4 | 21,8 ± 4,4[a] |
| France L2 | 0,7 ± 0,8 | 45,2 ± 9,0[a] |
| France L3 | 0,5 ± 0,5 | 63,0 ± 11,5[a] |
| France L4 | 0,0 ± 0,0 | 14,3 ± 3,2[a] |
| France L5 | 1,7 ± 1,1 | 55,8 ± 7,2[a] |
| Italy | 0,0 ± 0,0 | 97,7 ± 12,5[a] |
| Brazil | 0,2 ± 0,4 | 16,8 ± 5,3[a] |

[a] Statistically significant difference from negative control (Mann-Whitney U test, $p<0,05$).

Micronucleus frequency in root cells of negative controls is normally low

Micronucleus frequency of positive controls is significantly different from the control

**Table D.4 — Mitotic index (number of cells in division/1 000 cells) for the maleic hydrazide (1,12 mg/l) in liquid phase**

| | negative control | positive control |
|---|---|---|
| France L1 | 61,5 ± 5,2 | 24,8 ± 4,0 |
| France L2 | 88,0 ± 15,1 | 25,0 ± 9,7 |
| France L3 | 113,2 ± 9,3 | 92,3 ± 20,2 |
| France L4 | 29,2 ± 4,8 | 21,7 ± 4,0 |
| France L5 | 97,2 ± 13,5 | 77,9 ± 7,0 |
| Italy | 128,0 ± 10,4 | 23,0 ± 14,4 |
| Brazil | 37,5 ± 6,2 | 27,7 ± 5,3 |

## D.2 Contaminated soil results

See Tables D.5 to D.8.

A contaminated soil was sampled, sieved and sent to the laboratories: each participant performed tests with a direct exposure of plants to the soil, and with an exposure of plants to the aqueous extract of the soil. Participants prepared the aqueous contaminated soil extract according to ISO/TS 21268-2.

**Table D.5 — Micronucleus frequency (number of micronuclei/1 000 cells) according to the concentration of contaminated soil (direct exposure)**

| | Contaminated soil % | | |
|---|---|---|---|
| | **0** | **10** | **100** |
| France L1 | 0,2 ± 0,4 | 1,5 ± 1,0 | 5,5 ± 1,0[a] |
| France L2 | 0,0 ± 0,0 | 0,3 ± 0,8 | 20,8 ± 1,9[a] |
| France L3 | 0,2 ± 0,4 | 0,5 ± 0,5 | 1,5 ± 1,3 |
| France L4 | 0,0 ± 0,0 | 0,2 ± 0,4 | 0,3 ± 0,5 |
| France L5 | 0,7 ± 0,5 | 5,5 ± 1,0[a] | 16,0 ± 3,7[a] |
| Italy | 0,3 ± 0,8 | 3,3 ± 2,2[a] | 7,2 ± 2,9[a] |
| Brazil | 0,0 ± 0,0 | 4,8 ± 2,3[a] | 18,3 ± 3,6[a] |

[a] Statistically significant difference from negative control (Kruskal Wallis test, $p<0,05$).

**Table D.6 — Mitotic index (number of cells in division/1 000 cells) according to the concentration of contaminated soil (direct exposure)**

| | Contaminated soil % | | |
|---|---|---|---|
| | **0** | **10** | **100** |
| France L1 | 96,3 ± 8,7 | 89,5 ± 8,6 | 64,3 ± 6,1 |
| France L2 | 93,9 ± 15,1 | 119,8 ± 23,4 | 119,2 ± 17,7 |
| France L3 | 112,5 ± 20,5 | 103,7 ± 13,9 | 77,0 ± 17,3 |
| France L4 | 30,0 ± 7,5 | 34,7 ± 7,9 | 60,5 ± 14,3 |
| France L5 | 149,2 ± 16,4 | 184,8 ± 19,2 | 128,0 ± 10,4 |
| Italy | 139,2 ± 13,8 | 132,2 ± 17,8 | 147,0 ± 11,9 |
| Brazil | 32,7 ± 7,5 | 37,0 ± 8,9 | 31,5 ± 5,9 |

**Table D.7 — Micronucleus frequency (number of micronuclei/1 000 cells) according to the concentration of contaminated soil aqueous extract (indirect exposure)**

| | Soil aqueous extract % | | |
|---|---|---|---|
| | **0** | **10** | **100** |
| France L1 | 0,2 ± 0,4 | 5,8 ± 1,5[a] | 7,0 ± 1,8[a] |
| France L2 | 0,7 ± 0,8 | 2,3 ± 2,5 | 2,0 ± 1,4[a] |
| France L3 | 0,5 ± 0,5 | 1,2 ± 1,2 | 1,3 ± 1,4 |
| France L4 | 0,0 ± 0,0 | 0,0 ± 0,0 | 0,3 ± 0,8 |
| France L5 | 1,7 ± 1,1 | 14,0 ± 0,5[a] | 13,8 ± 4,5[a] |
| Italy | 0,0 ± 0,0 | 4,8 ± 2,9[a] | 8,7 ± 9,9[a] |
| Brazil | 0,2 ± 0,4 | 4,3 ± 2,1[a] | 16,7 ± 3,9[a] |

[a] Statistically significant difference from negative control (Kruskal Wallis test, $p<0,05$).

**Table D.8 — Mitotic index (number of cells in division/1 000 cells) according to the concentration of contaminated soil aqueous extract (indirect exposure)**

| | Soil aqueous extract % | | |
|---|---|---|---|
| | **0** | **10** | **100** |
| France L1 | 61,5 ± 5,2 | 44,3 ± 11,9 | 48,7 ± 4,0 |
| France L2 | 88,0 ± 15,1 | 106,2 ± 29,9 | 108,2 ± 10,2 |
| France L3 | 113,2 ± 9,3 | 107,3 ± 18,7 | 106,5 ± 10,0 |
| France L4 | 29,2 ± 4,8 | 28,8 ± 4,9 | 38,3 ± 10,9 |
| France L5 | 97,2 ± 13,5 | 90,6 ± 18,5 | 93,7 ± 13,8 |
| Italy | 128,0 ± 10,4 | 118,7 ± 13,6 | 111,0 ± 11,8 |
| Brazil | 37,5 ± 6,2 | 32,3 ± 7,4 | 40,0 ± 6,9 |

Results are different according to the laboratories, probably due to variabilities in reference soil (France L4 used a reference soil with high amount of clay and silt instead of a sandy soil), exposure duration (France L3 used 96 h exposure duration instead of 48 h) and the way to prepare the slides: some participants performed the *Vicia-micronucleus* test for the first time (France L3 and France L4). Moreover, each participant had to prepare its own soil aqueous extract, which may also be a variability source.

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