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Soil quality — Determination of the toxic effects of pollutants on germination and early growth of higher plants

Qualité du sol — Détermination des effets toxiques des polluants sur la germination et la croissance primaire des plantes supérieures

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

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

Introduction

Ecotoxicological testing of test soils or waste materials to be disposed on soil are required to assess the potential environmental risk resulting from soil pollution or the disposal of wastes such as sewage sludge on farmland. There is also a need to monitor the quality of soil after reclamation of industrial sites. Therefore, a very practical and rapid germination and growth test has been developed based on seed germination and seedling growth in controlled environmental conditions.

The assay, which does not require any pretreatment of the seeds, is performed in “transparent test plates”, incubated vertically, to allow the roots and the shoots of the germinated seeds to be seen. After 72 h exposure, a picture of the transparent test plates is taken and can be analysed “by image analysis” for multiple endpoints, such as percentage of seed germination and of length of roots and shoots. To account for the plant species variability in sensitivity, the assays are performed with the seeds of three plant species: one monocotyl (*Sorghum saccharatum*) and two dicotyls (*Lepidium sativum* and *Sinapis alba*).

A major advantage of this test is that after the shooting and storing of the pictures of the test plates, the measurements by image analysis can be postponed to any appropriate timing.

Reference or standard soils can be used as negative controls, such as, for example, the ISO standard artificial soil according to ISO 11269-1 and ISO 11269-2.

Commercially available seeds, with a shelf life longer than one year, allow the use of this test at any time of the year.

Two International interlaboratory comparisons demonstrated that the test provides good results.

A substantial number of studies report data on the application of this test on various types of soils and soil materials with several types of plant species.

Soil quality — Determination of the toxic effects of pollutants on germination and early growth of higher plants

1 Scope

This International Standard describes a technique for determining the effects of soil and soil-related materials on the seed germination and early growth of higher plants. These endpoints are useful indicators for the assessment of the quality of a soil as a habitat for organisms. This International Standard is applicable to all soils in which soil organisms are active and may be used to evaluate:

— the effects on plants due to toxicity of solid or liquid chemicals contaminating soil or materials (compost, sludge, waste) and chemicals added to soil;

— the changes in the soil effect on plants after restoration measures.

2 Normative references

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 11269-1, *Soil quality — Determination of the effects of pollutants on soil flora — Part 1: Method for the measurement of inhibition of root growth*

ISO 11269-2, *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/TS 20281, *Water quality — Guidance on statistical interpretation of ecotoxicity data*

3 Terms and definitions

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

3.1
artificial soil
mixture of sand, kaolinite, peat and calcium carbonate prepared according to ISO 11269-1 and ISO 11269-2

3.2
control soil
reference or standard soil used as a control and as a medium for preparing dilution series with test soils or a reference substance

3.3
reference soil
uncontaminated site-specific soil (e.g. collected in the vicinity of a contaminated site) with similar properties (nutrient concentrations, pH, organic carbon content and texture) as the test soil

3.4
standard soil
field-collected soil or artificial soil whose main properties (e.g. pH, texture, organic matter content) are within a known range

Note 1 to entry: The properties of standard soils can differ from the test soil.

EXAMPLE Euro-soils[1], artificial soil[2], LUFA soil.[1)]

3.5
test soil
either a natural or an artificial clean soil that is spiked with the test substance or a contaminated natural soil (a site soil)[5]

3.6
seeding emergence
appearance of a visible seedling above the surface of the cover material

[SOURCE: ISO 17126:2005, 3.1, modified]

3.7
germination
appearance of a root of at least 1 mm of length

3.8
pure water
grade of water, produced, for example, by single distillation, by de-ionization, by ultra-filtration or by reverse osmosis[5]

3.9
root length
length of the root from seed to root tip

3.10
shoot length
length of the part that grows upward, from seed to tip

3.11
water saturation
maximum water content that a soil can retain against gravity under undisturbed soil conditions, conventionally stated as water content two days to three days after full saturation with water

[SOURCE: ISO 11074:2015, 2.1.5 field capacity, modified]

3.12
water saturated soil
soil which has reached its maximum water content

3.13
water-holding capacity
mass of water that evaporates from soil saturated with water when the soil is dried to constant mass at 105 °C, divided by the dry mass of the soil[7]

3.14
negative control
any well-characterized material or substance that, when tested by a specific procedure, demonstrates the suitability of the procedure to yield a reproducible, appropriately negative, non-reactive or minimal response in the test system

[SOURCE: ISO 10993-10:2010, 3.12, modified]

3.15
effect percentage
percentage decrease of the seed germination and the growth of the plant roots and/or shoots in the test soil in comparison to the control soil

1) Euro-soils, artificial soil and LUFA soil are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

4 Principle

This method compares the seed germination and early growth of monocotyledonous and dicotyledonous plants in a test soil and/or a series of mixtures with a control soil. This method may also be used for the testing of compost, sludge or waste.

Seeds of one monocotyledonous plant, such as *Sorghum saccharatum* (L.) Moench, and two dicotyledonous plants, such as *Lepidium sativum* L. and *Sinapis alba* L., are exposed to the test material under controlled conditions. After (72 ± 1) h, the number of germinated seeds is recorded and the length of the roots of the test plants is measured in the test soil and in the control soil.

If different seed species are used, the length of the incubation period may be adjusted, depending on the time of germination of the seeds and the growth speed of the roots.

The test makes use of unique flat and shallow transparent test plates (6.3) composed of two compartments, the lower one of which contains hydrated soil.

Seeds of the selected test plants are positioned at equal distance near the middle ridge of the test plate (6.3) on a black filter paper (6.5) placed on top of the hydrated soil.

After closing the test plates (6.3) with their transparent cover, the test plates are placed vertically in a holder (6.4) and incubated at (25 ± 1) °C for (72 ± 1) h.

At the end of the incubation period, the length of each root (and shoot, if wished) can be measured directly with a ruler and recorded.

Alternatively, a “digital” picture is taken of the test plates (6.3) with the germinated plants (either with a digital camera, a webcam camera or a flatbed paper scanner) for storage in a computer file. The subsequent root length measurements are performed by image analysis. The analyses on germination and root growth can then be made immediately or postponed to any appropriate timing.

NOTE The same procedure can be applied to also measure the shoot height, if wished. Calculation of the shoot/root length ratio is a possible additional effect parameter.

5 Reagents, test organisms and media

5.1 Water.

Pure water having a conductivity below 10 µS/cm.

5.2 Test organisms.

The test organisms are seeds of one monocotyledonous plant, such as *Sorghum saccharatum* (L.) Moench, and two dicotyledonous plants, such as *Lepidium sativum* L. and *Sinapis alba* L.

Investigations have been performed not only with the three plant species indicated in 5.2 but also with other monocotyl and dicotyl plant species. A synthesis on these published studies is given in Annex A.

Seeds coated with insecticides and/or fungicides should be avoided.

5.3 Control soil.

Either reference or standard soils can be used as the control soil, if unhindered growth of the test plants in these soils can be expected.

When comparing the root elongation in 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 contaminant may lead to differences in root lengths and may induce false positive test results.

Alternatively, artificial soil according to ISO 11269-1 and ISO 11269-2 may be used. The substrate called "artificial soil" has the following composition:

	Percentage expressed on dry-mass basis
Sphagnum peat finely ground and with no visible plant remains	10 %
Kaolinite clay containing not less than 30 % kaolinite	20 %
Industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 mm to 0,2 mm)	69 %

NOTE As indicated in ISO 11269-1, 5 % peat have proven to be sufficient for maintaining the desired structure of the artificial soil (with a corresponding increase of the sand percentage to 75 %). A lower percentage of kaolinite clay (10 % instead of 20 %) is furthermore very close to the clay content of LUFA 2.2 and hence more representative for a natural soil. The following composition of the artificial soil is therefore recommended: peat 5 %, kaolinite clay 10 % and sand 85 %.

Approximately 0,3 % to 1,0 % calcium carbonate ($CaCO_3$, pulverized, analytical grade) is necessary to get a pH of 6,0 ± 0,5.

6 Apparatus and materials

6.1 Incubator or temperature controlled room, suitable for maintaining the specified conditions at (25 ± 1) °C.

6.2 Digital camera, webcam camera or flatbed paper scanner, to shoot pictures of the test plates with the germinated seeds, for storage in a computer file.

6.3 Test plates, transparent plates in polyvinylchloride (PVC).

The test plates are composed of a bottom part separated by a middle ridge into an upper part and a lower part and a flat cover. Test plates can be "handmade" with the aid of transparent PVC sheet and small rectangular sticks as described in Annex B.

Alternatively, commercially available[2)] test plates can be used. The lower part of these test plates is intended to hold approximately 90 cm^3 of test soil. The latter test plates have on both parts on their side small rectangular cavities for closing the plates tightly by a unique click system. The test plates shall be provided with a label to record the specifics of each test plate (type of soil, type of seed, number of the replicate).

2) The test plates supplied by MicroBioTests Inc. Mariakerke-Gent, Belgium 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.

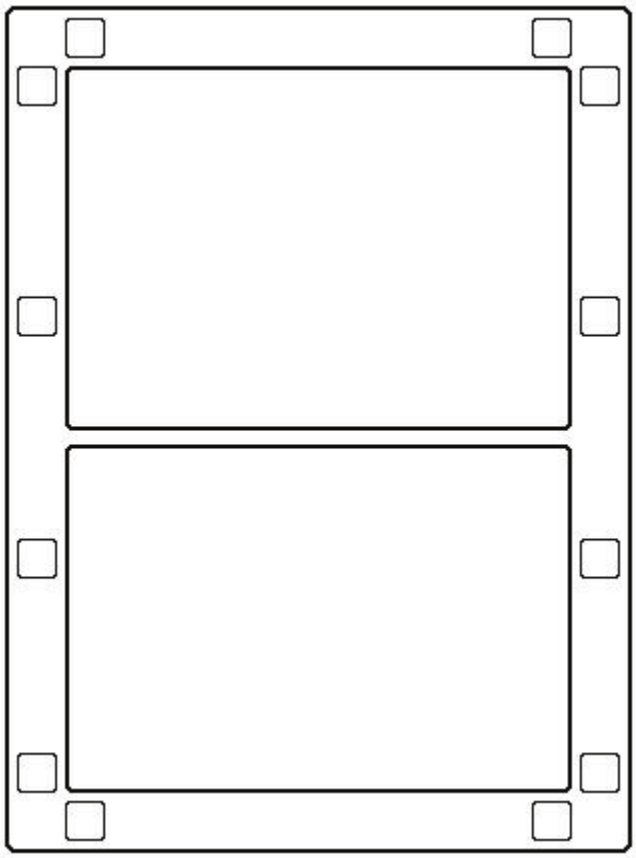

Figure 1 — Test plate with cavities on the side for tight closing of the plate

6.4 Test plate holders, cardboard holders for vertical incubation of six test plates (6.3) each.

6.5 Black filter papers, rectangular high purity black filter papers (e.g. 85 g/m^2, 0,17 mm thickness, 45 s filtration speed) fitting the lower part of the test plate, to be placed on top of the soil in the lower compartment of the test plates (6.3).

6.6 Microsieve cylinder, small plastic cylinder provided at the bottom with a nylon gauze, to be used for determination of the water to be added to the test soil.

6.7 Wide mouth micropipette, plastic micropipette to be used with the microsieve cylinder (6.6) for determination of the water to be added to the test soil.

6.8 Sieve, sieve of 2 mm mesh for sieving the test soil prior to use for the tests.

6.9 Thin spatula, hand tool with a thin blade used to mix the test soil with water.

6.10 Flat spatula, hand tool with a broad, flat blade that is used to spread and flatten the test soil into the lower compartment of the test plates (6.3).

6.11 Tweezers, small pincer-like tool for handling the seeds.

7 Treatment and preparation of samples

7.1 Soil samples

The assays are carried out at water saturation of the soils at the start of the tests.

7.1.1 Water-holding capacity determination

The water saturated soil can be obtained by adding to the soil mass the appropriate volume of pure water determining the water-holding capacity.

7.1.2 Alternative procedure for determination of the volume of water to be added in the test plates for hydration of air-dried soils

A simple and quick alternative procedure can be used to determine the volume of water to be added to air-dried soils in the test plates (6.3).

For the artificial soil recommended in 5.3, the amount of water needed to be added for hydration has been determined experimentally. Based on a water/soil ratio (on a vol/vol basis) of 0,39, one needs to add 35 ml pure water to the 90 cm^3 control soil in the test plate (6.3).

For other control soils, the amount of water needed to be added for hydration needs to be experimentally determined, following the procedure indicated in the next paragraph for test soil samples.

For test soil samples, they first need to be air-dried, then the dry soil is sieved through a sieve (6.8) to eliminate all coarse material.

NOTE Air drying is requested only on a separate soil sample for subsequent determination of the volume of water to be added in the test plates.

Mix thoroughly 50 ml pure water and 90 cm^3 test soil in a beaker with the aid of a thin spatula (6.9). After 1 min to 2 min, two layers show up: the hydrated soil and a layer of water on top.

Lower vertically the microsieve cylinder (6.6) into the beaker, down to the surface of the hydrated soil, and then lower it a little further down, so that it starts filling with supernatant.

With the wide mouth micropipette (6.7), suck up the water inside the microsieve and transfer it into a graduated cylinder.

Put the microsieve cylinder (6.6) again into the beaker, and push it down a little further, so that it takes up additional water from the soil. Transfer again the recovered water in the graduated cylinder and repeat the former manipulations until no water comes out anymore from the soil.

Calculate how much water was needed for a complete hydration of the test soil. This volume (Vsat) is the volume of water that has originally been added to the soil (= 50 ml) minus the volume of supernatant water (S) which has been recovered in the graduated cylinder (Vsat = 50 − S).

8 Procedure

The procedure described hereafter is intended for use of 90 cm^3 soil in the test plates (6.3).

In case of assessment of the dose-response relationship on an unknown solid sample, a full test shall be performed comprising a number of mixtures of the control soil with the sample (e.g. compost, sludge, waste). The dilutions shall be prepared within a geometric series with a separation factor not exceeding 2,0 (see ISO 11269-1). According to the selected dilution range, the test soil is mixed with the reference soil or the standard soil thoroughly (either manually or by using a hand mixer). The homogeneity of the mixture is checked visually. For each of the resulting dilutions, the volume of water to be added in the test plates (6.3) shall be experimentally determined (7.1.2).

8.1 Test procedure for determination of the effects of contaminated soils

8.1.1 Addition of control soil and test soil to the test plates and hydration of the soils

8.1.1.1 Control soil

If the artificial soil (5.3) is used as control, put 90 cm^3 control soil (5.3) in the lower compartment of a test plate (6.3), then slowly drop 35 ml pure water over the whole surface of the control soil in the test plate.

If another control soil is used, add the amount of water experimentally determined (7.1.2).

Wait 1 min to 2 min so that the water hydrates the soil totally.

With the flat blade spatula (6.10), flatten the wet soil evenly over the total surface of the bottom compartment of the test plate (6.3), in order to obtain a layer of uniform depth.

Repeat the former operations for all the negative control test plates (6.3) (= nine test plates).

8.1.1.2 Test soil

Put 90 cm^3 of sieved test soil into the bottom compartment of a test plate (6.3), then hydrate the test soil as indicated above for the control soil by dropping a volume of pure water equal to Vsat (7.1.2) on the surface of the soil in the test plate and flattening it with the flat blade spatula (6.10).

Repeat the former operations for all the other plates with test soil (= nine test plates).

NOTE An alternative procedure to measuring repeatedly a volume of 90 cm^3 of soil in a graduated vessel is to determine the weight of test soil corresponding to 90 cm^3 and prepare such weights for transfer into the corresponding test plates (6.3).

8.1.2 Placing of the seeds

Place on top of the hydrated soil one black filter paper (6.5) in all the test plates (6.3), to avoid trapping air bubbles under the filter, and wait 1 min to 2 min until the filter is totally wet.

The assay is performed in three replicates with three different seeds. This means that three test plates (6.3) with control soil, and three test plates with test soil, shall be inoculated with each type of seed.

With the aid of tweezers (6.11), take 10 seeds of the same test plant and put them in one row on the black filter paper (6.5) at equal distance of each other, and at about 1 cm from the middle ridge of the test plate (6.3).

Put the cover part of the test plate on the bottom part, close the test plate tightly and label it indicating the specifics of the test (type of soil, type of seed, number of the replicate).

Repeat the former operation for each seed for the three control test plates and the three replicates with test soil.

8.1.3 Incubation of the test plates

Place the six test plates (6.3) inoculated with the same seed (three with test soil and three with control soil) vertically in one test plate holder (6.4).

Repeat this operation for the two other seeds.

Put the three test plate holders (6.4) with their test plates (6.3) in the incubator (6.1) and incubate at (25 ± 1) °C for (72 ± 1) h.

NOTE Light does not seem to have an influence on the germination of the seeds nor the growth of the roots during the short (three days) incubation time. It is therefore advised not to provide illumination in the incubator during the test period.

8.1.4 Image recording

The picture of the test plates at the end of the exposure period can be taken either with a webcam camera, a digital camera or a flatbed paper scanner. The picture shall then be transferred to a file in a computer.

Any type of image analysis programme can be used for the subsequent analyses, provided it allows for length measurements.

NOTE A convenient and practical image analysis programme is "Image J" which can be downloaded directly from the Internet (http://rsb.info.nih.gov/ij/download.html).

9 Measurement

All the measurements should be made within (60 ± 10) min after the end of exposure period or can be postponed to any appropriate timing by image recording (8.1.4) of the test plates (6.3).

9.1 Counting the number of germinated seeds

Count the number of germinated seeds, N_S, in the test plates (6.3) and record the figure on the results sheet "Individual data".

9.2 Measurement of the root lengths of the germinated seeds

9.2.1 Measurement of all the root lengths in each test plate

9.2.1.1 Procedure for direct visual length measurement

Transfer one germinated seed on a black paper sheet.

With the aid of the tweezers (6.11), stretch the root and measure its length LR to the closest millimetre with a ruler. Record the figures for all the roots on the results sheet "individual data".

Repeat the procedure for all seeds of a test plate (6.3) and for all the test plates.

The same procedure may be applied to measure the shoot height, in which case the shoot/root length ratio can be calculated.

9.2.1.2 Procedure for image analysis of length measurement

Open the image analysis programme which allows to make length measurements.

Open the file with the saved pictures of the test plates (6.3) and select one of the saved pictures.

Calibrate the measurement unit for length measurement of the roots (in millimetres) following the Image Analysis programme instructions.

Perform the length measurement LR on all the roots of the germinated seeds in the test plates (6.3), record each length LR on the result sheet "individual data" (see Table 1).

Repeat these operations for all the test plates (6.3).

Table 1 — Result sheet "individual data" (for each plant species)

Seed number	Control 1	Test soil 1	Control 2	Test soil 2	Control 3	Test soil 3
1	LR1	LR1	LR1	LR1	LR1	LR1
2	LR2	LR2	LR2	LR2	LR2	LR2
3	LR3	LR3	LR3	LR3	LR3	LR3
4	LR4	LR4	LR4	LR4	LR4	LR4
5	LR5	LR5	LR5	LR5	LR5	LR5
6	LR6	LR6	LR6	LR6	LR6	LR6
7	LR7	LR7	LR7	LR7	LR7	LR7
8	LR8	LR8	LR8	LR8	LR8	LR8
9	LR9	LR9	LR9	LR9	LR9	LR9
10	LR10	LR10	LR10	LR10	LR10	LR10
Mean root length in each test plate	M_{LR}	M_{LR}	M_{LR}	M_{LR}	M_{LR}	M_{LR}
Number of germinated seeds in each test plate	N_S	N_S	N_S	N_S	N_S	N_S

9.2.2 Measurement of the length of the longest root in each test plate

Substantial time saving can be made by measuring only the length of the longest root LLR in each test plate instead of measuring the length of all the roots.

NOTE The International interlaboratory comparisons referred to in Clause 11 have shown that this method basically gives the same outcome for the assay as that of the measurement of all the roots in each test plate (see Annex C).

Record the lengths of the longest root (LLR) in each test plate (6.3) on the results sheet "longest root" (see Table 2).

Table 2 — Result sheet "longest root" (for each plant species)

Length longest root	Control	Test soil
Test plate 1	LLR	LLR
Test plate 2	LLR	LLR
Test plate 3	LLR	LLR
Mean length longest root for the three replicate test plates	M_{LLR}	M_{LLR}

10 Calculation of the percentage inhibition

With the data recorded in the results sheet "individual data", calculate for each test plate (6.3) the following:

— the mean (M_{LR}) of the root length LR and report the figures in the result sheet "individual data";

— and/or the mean (M_{LLR})of the length of the longest root LLR (which then has to be reported in the result sheet "longest root").

Then calculate the following for each plant:

— the mean (M_S) of the 3 N_S;

— the mean (MM_{LR}) of the 3 M_{LR};

and report the data in the result sheet "mean data" (see Table 3).

Table 3 — Result sheet "mean data" (for each plant species)

	Control	Test soil
Mean number of germinated seeds in the three replicate test plates	M_S	M_S
Mean root length in the three replicate test plates	MM_{LR}	MM_{LR}

Calculate the mean (M_{LLR}) of the three LLR and report the data in the result sheet "longest root measurement".

Calculate the percentage inhibition of seed germination and root growth inhibition for each plant using Formula (1):

$$\frac{(A-B)}{A} \times 100 \quad (1)$$

where

A is the mean seed germination (M_S) or root length (MM_{LR}) or longest root length (M_{LLR}) in the control soil;

B is the mean seed germination (M_S) or root length (MM_{LR}) or longest root length (M_{LLR}) in the test soil.

The statistical analysis of the data should be done according to the indications of ISO/TS 20281.

11 Reference chemical

It is recommended that a test be carried out regularly with a reference chemical in order to demonstrate the uniformity of the laboratory test conditions. Boric acid (H_3BO_3) which is one of the two reference chemicals used for ISO standards on plant tests (ISO 11269-1 and ISO 11269-2) was also selected for this test and was used for an International interlaboratory comparison (see Clause 12 and Annex C).

12 Precision

In 2011, 28 laboratories from 12 countries participated in an International interlaboratory comparison on this test, measuring the mean % inhibition of the mean root length (and also of the mean of the longest root) for the assay with 250 mg boric acid per kg control soil. The interlaboratory comparison revealed that at a concentration of 250 mg boric acid/kg control soil, there is no inhibition of the germination of the seeds.

The results on the mean root lengths, the mean length of the longest roots and the effect percentages obtained by the participants are summarized in Annex C.

In 2015, eight laboratories from six countries participated in a second International interlaboratory comparison, measuring the mean % inhibition of the mean root length and the mean of the longest root for assays with two soils (SOIL 1 and SOIL 2) and one waste (WASTE SOIL). The latter actually is mixture of 75 % LUFA soil and 25 % WOO waste (a preserved wood waste contaminated with copper and other heavy metals) as the original WOO waste was "extremely toxic" (100 % of effect) to the three test plant test. The natural soil LUFA 2.2 was selected as negative control (CONTROL 2) for this International interlaboratory comparison.

The results from this second International interlaboratory comparison are also summarized in Annex C.

13 Validity criteria

On the basis of the International interlaboratory comparisons on the phytotoxicity test reported in Clause 11, the following validity criteria have been selected:

— 70 % of the seeds of the three test species shall have germinated in the negative controls at the end of the three days exposure period;

— the mean root length in the negative controls shall be at least 30 mm for the three test species and 40 mm in case the measurements are based on the mean length of the longest root.

14 Test report

The test report shall include the following information:

a) a reference to this International Standard, i.e. ISO 18763;

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

c) information about the test plant species (Linnaean classification, variety, source);

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

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

f) the characteristics of the control soil;

g) the number of germinated seeds of each plant;

h) the length of the roots of each plant and the corresponding mean value;

i) alternatively, the length of the longest root of each plant, if this result is preferred over the mean value of the length of the roots of each plant;

j) the shoot height (if measured);

k) the shoot/root length ratio (if shoot length is measured);

l) any other effects observed;

m) the results of the test (in the form of a table) for each test plant (indicate whether growth inhibition is statistically significant or the level of significance of any growth inhibition observed).

Annex A
(informative)

Application of the phytotoxicity test in transparent test plates on natural and artificial soils and soil materials, with different plant species

A summary is given in Table A.1 of the major characteristics of 28 publications which report application of the phytotoxicity test in transparent test plates on various types of natural or artificial soils and soil materials.

The numbers in the first column of the table refer to the specific publication of which the complete reference is given in the Bibliography.

The other columns of the table briefly indicate the type of substrate (i.e. soil or soil material used in the study), the treatment which has either or not been applied to the substrate (e.g. spiking with chemicals, or mixing with other solids or wastes) and the scientific names of the plant species which have been used for the specific studies.

Table A.1 — Bibliographic references on the use of the phytotoxicity test

Reference	Type of substrate	Substrate treatment	Plant test species
[9]	Artificial soil (sand, vermiculite, peat)	Spiking with sulfamethazine	*Lupinus luteus, Pisum sativum, Lens esculenta, Glycine max, Vigna angularis, Medicago sativa*
[10]	Contaminated soils (pesticides)	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[11]	Reference OECD soil	Spiking with treated effluents from wastewater treatment plants	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[12]	Natural light and heavy soils (weaky, loamy and silt clay)	No	*Zea mays*
[13]	Oil shale combustion fly ash	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[14]	Peat	Spiking with trichloroacetic acid	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[15]	Composts (bark material)	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[16]	River sediments	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[17]	River sediments	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[18]	Reference OECD soil	Spiking with cadmium and zinc sulfate	*Brassica napus, Sinapis alba, Spinacia oleracea*
	River sand		
[19]	River sediments	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
	Natural soil		
[20]	Contaminated sediments (by mining activities)	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[21]	Contaminated soils (by metals)	Electrokinetic metal decontamination	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*

Table A.1 *(continued)*

Reference	Type of substrate	Substrate treatment	Plant test species
[22]	Contaminated sediments (organic compounds and heavy metals)	Spiking with adsorbents	*Lepidium sativum*
[23]	Contaminated soils (metals)	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[24]	Contaminated sediments (by mining activities)	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[25]	Composts (from sewage sludges)	Mixing with standard OECD soil	*Lepidium sativum*
[26]	Contaminated soils (PAH hydrocarbons)	No	*Lepidium sativum*
[27]	Natural soils (sandy or loamy)	Addition of sewage sludge	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[28]	Sewage sludge-fly ash mixtures	Different ratios of sewage sludge and fly ash	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[29]	Solid wastes	No	*Avena sativa, Brassica rapa*
[30]	Contaminated soils (by heavy metals)	No	*Lepidium sativum*
[31]	Natural soils (sand, peat, clay)	Addition of sewage sludge	*Zea mays*
[32]	Sewage sludge	Addition of fly as hand/or lime	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[33]	Natural soil	Spiking with herbicides	*Sinapis alba, Fagopyrum esculentum, Cucumis sativus*
[34]	Natural soil	Spiking with herbicides	*Helianthus annuus, Cucumis sativus, Fagopyrum esculentum*
[35]	Contaminated river sediments	No	*Sinapis alba*
[36]	Artificial soil	Spiking with boric acid	*Trifolium pratense*
	Contaminated clay loam soil	No	*Hordeum vulgare, Elymus lanceolatus, Medicago sativa, Cucumis sativus, Sinapis alba, Sorghum saccharatum*
[36]	Solid wastes	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[37]	Natural soils	Spiking with diesel oil	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*
[38]	Wood based substrates	No	*Lepidium sativum, Sinapis alba, Sorghum saccharatum*

Annex B
(informative)

Assemblage of test plates for the phytotoxicity test

B.1 General

Test plates for the phytotoxicity test can be “handmade” in any dimensions.

The sizes of the materials listed hereunder are specified for a test plate in which 90 cm^3 of (hydrated) soil is put in the lower part of the bottom compartment of the plate (as described in the test procedure (Clause 8) of this test).

B.2 Materials needed

The following are the needed materials:

— two rectangular pieces of transparent PVC sheet (or thick transparency film) of a length of 193 mm and a width of 140 mm;

— three square plastic or wood strips of 8 mm thickness and 124 mm length;

— two square plastic or wood strips of 8 mm thickness and 193 mm length;

— one tube of a glue specific for gluing plastic or wood on PVC.

B.3 Assemblage procedure of the test plate

Put a thin amount of glue over the entire length of one side of the two long strips and of two of the shorter strips.

Place and glue these strips on the outer edges of one of the PVC sheets, in order to obtain a rectangular internal compartment of 178 mm × 124 mm × 8 mm.

Put a thin amount of glue over the entire length of one side of the third short stick and place and glue this strip transversally exactly in the middle of the test plate to divide it in two compartments of the same dimensions.

Once the glue has dried, the lower compartment of the test plate can be filled with 90 cm^3 of (hydrated) soil.

The further procedure, i.e. placing of the black filter paper and the seeds, is similar to the procedure described for the toxicity test.

The test plate is closed by placing the second PVC sheet on top of the bottom part and is kept tightly closed by sliding two (thin) rubber bands over the plate, near the top and the bottom.

Annex C
(informative)

International interlaboratory comparisons on the phytotoxicity test

In 2011, twenty-eight laboratories from twelve countries participated in an International interlaboratory comparison on this test, measuring the mean % inhibition of the mean root length (and also of the mean of the longest root) for the assay with 250 mg boric acid (BORIC ACID) per kg control soil. The artificial soil (5.3) was used as negative control (CONTROL 1).

In 2015, eight laboratories from six countries participated in a second International interlaboratory comparison, measuring the mean % inhibition of the mean root length and the mean of the longest root for assays with two contaminated natural soils (SOIL 1 and SOIL 2) and one waste (WASTE SOIL). The latter is a preserved wood waste which was, however, found that toxic that no germination occurs with any of the three test species. It was therefore decided to mix it the standard soil LUFA 2.2, which was selected as negative control (CONTROL 2), for this interlaboratory comparison. A 75 % LUFA soil and 25 % waste was eventually prepared for the ring test and was named WASTE SOIL.

The characteristics and the chemical composition of the two contaminated natural soils and the waste can be found in References [40], [41], [42] and that of the LUFA 2.2 standard soil in the Data Sheet for Standard Soils of the "Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer".

The validity criteria have been established on the basis of the results of the first International interlaboratory comparison. Therefore, for this comparison, the results of all 28 participants were submitted to the statistical analysis for the detection of outliers and the calculation of the general mean, $\bar{X}$, the repeatability and the reproducibility variances, s_r and s_R, according to ISO 5725-2.

For the second International interlaboratory comparison, the selected validity criteria were applied. All eight laboratories met these criteria for *Lepidium sativum* and *Sinapis alba*, while one laboratory failed to meet two of the criteria for *Sorghum saccharatum*. Therefore, eight valid results were obtained for the dicotyl species and seven for the monocotyl. These data too were submitted to the statistical analysis according to ISO 5725-2.

The results of the two International interlaboratory comparisons are summarized in Tables C.1 to C.3.

In both comparisons, considering the valid results, a high percentage of germination has been obtained by all the participants for the three test species, both in the control test plates and in those with samples (BORIC ACID, SOIL 1, SOIL 2 and WASTE SOIL).

In the second International interlaboratory comparison, SOIL 1 did not show toxicity on neither the two dicotyls nor on the monocotyl test species; SOIL 2 in turn produced a significant inhibition of both root length and longest root length of the three plant species; WASTE SOIL was not found toxic to *Lepidium sativum*, but had a slight (but significant) toxic effect on the root growth of *Sinapis alba*, and a slight (but not statistically significant) effect on the root length of *Sorghum saccharatum*.

Tables C.4 and C.5 report the percentages of effect for BORIC ACID (first comparison), SOIL 2 and WASTE SOIL (second comparison).

The repeatability and reproducibility percentages of the mean root length and the mean of the longest root are quite low (mostly below 20 %) and hence very satisfactory.

Furthermore, the second interlaboratory comparison also confirms the findings of the first ring test, namely that the measurement of the longest root length of the three replicate test plates provides similar results as the measurement of the root lengths of all the roots in each test plate: in fact, in the first International interlaboratory comparison, the coefficient of determination for the correlation between the mean root length and the longest root length, based on a total of 51 data pairs, explains

93 % of the data variability; in the second International interlaboratory comparison, with a total of 25 data pairs, the explained variability is 88 %.

The measurement of only the longest root instead of that of all the roots as an effect criterion is actually a substantial saving of time.

Finally, the second ring test also showed that the validity criteria in the ISO/DIS proposal, namely 70 % seed germination, and a minimum of 30 mm root length and 40 mm length for the longest roots in the controls, are meaningful acceptability criteria for this phytotoxicity test.

Table C.1 — Interlaboratory test results for the mean number of germinated seeds

	N	n_o	$\bar{X}$	s_r	CV_r	s_R	CV_R
Lepidium sativum							
CONTROL 1	26	2	9,2	0,8	8,5	0,8	8,9
BORIC ACID	27	1	9,4	0,6	6,8	0,7	7,8
CONTROL 2	8	0	9,4	0,7	7,9	0,7	7,9
SOIL 1	5	3[a]	9,9	0,4	3,7	0,4	3,7
SOIL 2	7	1	9,7	0,4	4,5	0,5	5,0
WASTE SOIL	7	1	9,9	0,4	3,8	0,4	3,8
Sinapis alba							
CONTROL 1	26	2	9,4	0,7	7,9	0,8	8,5
BORIC ACID	23	5	9,6	0,5	4,9	0,6	6,1
CONTROL 2	8	0	9,5	0,7	7,2	0,7	7,2
SOIL 1	6	2	9,7	0,7	6,9	0,7	6,9
SOIL 2	8	0	9,4	0,8	8,1	0,8	8,1
WASTE SOIL	6	2	9,4	0,5	5,6	0,7	7,6
Sorghum saccharatum							
CONTROL 1	25	3	9,2	0,7	7,6	0,8	8,3
BORIC ACID	25	3	9,0	0,8	8,7	0,8	8,8
CONTROL 2	7	0	8,5	1,2	13,5	1,2	13,5
SOIL 1	6	1	8,4	1,2	14,2	1,2	14,2
SOIL 2	6	1	8,3	1,5	18,3	1,5	18,3
WASTE SOIL	7	0	8,4	1,1	13,0	1,4	17,0

N is the number of laboratories after elimination of outliers.

n_o is the number of outliers.

$\bar{X}$ is the mean number of germinated seeds.

s_r is the standard deviation of repeatability.

CV_r is the variation coefficient of repeatability, in %.

s_R is the standard deviation of reproducibility.

CV_R is the variation coefficient of reproducibility, in %.

[a] This is a statistical artefact, with no practical relevance. In fact, the mean number of germinated seeds for the eight laboratories (including the statistical outliers) is 9,4, as for CONTROL 2.

Table C.2 — Interlaboratory test results for the mean root length

	N	n_o	$\bar{X}$	s_r	CV_r	s_R	CV_R
Lepidium sativum							
CONTROL 1	28	0	51,0	5,8	11,3	8,3	16,3
BORIC ACID	27	1	27,7	3,3	11,9	4,7	17,1
CONTROL 2	8	0	56,2	3,6	6,3	5,8	10,2
SOIL 1	8	0	57,7	3,3	5,7	8,1	14,0
SOIL 2	7	1	33,6	2,4	7,1	3,7	11,1
WASTE SOIL	8	0	53,9	4,1	7,6	4,8	8,9
Sinapis alba							
CONTROL 1	26	2	50,4	5,1	10,1	9,3	18,4
BORIC ACID	27	1	28,2	3,4	12,1	5,6	19,8
CONTROL 2	8	0	59,5	7,3	12,2	9,9	16,6
SOIL 1	8	0	60,9	5,8	9,5	9,8	16,2
SOIL 2	7	1	33,6	4,1	12,1	4,8	14,3
WASTE SOIL	8	0	47,0	5,8	12,2	8,0	17,0
Sorghum saccharatum							
CONTROL 1	28	0	47,7	7,2	15,2	14,7	30,7
BORIC ACID	25	3	32,4	2,9	8,8	6,7	20,6
CONTROL 2	7	0	33,2	4,5	13,5	4,5	13,5
SOIL 1	6	1	33,2	3,3	10,0	4,9	14,7
SOIL 2	7	0	21,9	4,7	21,6	5,8	26,5
WASTE SOIL	7	0	26,5	4,1	15,6	8,0	30,3

N is the number of laboratories after elimination of outliers.

n_o is the number of outliers.

$\bar{X}$ is the mean root length, in mm.

s_r is the standard deviation of repeatability.

CV_r is the variation coefficient of repeatability, in %.

s_R is the standard deviation of reproducibility.

CV_R is the variation coefficient of reproducibility, in %.

Table C.3 — Interlaboratory test results for the mean longest root length

	N	n_o	$\bar{X}$	s_r	CV_r	s_R	CV_R
Lepidium sativum							
CONTROL 1	27	1	65,0	4,9	7,6	8,8	12,6
BORIC ACID	27	1	38,3	4,1	10,6	4,9	12,8
CONTROL 2	8	0	71,8	4,1	5,7	6,1	8,4
SOIL 1	7	1	75,3	3,2	4,2	7,6	10,1
SOIL 2	8	0	38,0	3,5	9,2	9,7	25,6
WASTE SOIL	8	0	67,8	3,6	5,3	4,9	7,3
Sinapis alba							
CONTROL 1	27	1	78,6	9,3	11,9	19,6	25,0
BORIC ACID	26	2	46,1	4,1	8,8	8,3	17,9
CONTROL 2	8	0	82,5	4,2	5,1	7,3	8,9
SOIL 1	7	1	80,3	4,6	5,8	8,2	10,3
SOIL 2	6	2	45,7	4,2	9,1	4,2	9,1
WASTE SOIL	8	0	71,0	6,1	8,6	6,1	8,6
Sorghum saccharatum							
CONTROL 1	27	1	78,6	9,3	11,9	19,6	25,0
BORIC ACID	26	2	46,1	4,1	8,8	8,3	17,9
CONTROL 2	6	1	49,5	6,9	14,0	7,4	14,9
SOIL 1	6	1	53,7	6,6	12,3	12,2	22,8
SOIL 2	7	0	31,5	5,7	18,1	9,0	28,6
WASTE SOIL	6	1	47,2	5,7	12,0	11,2	23,7

N is the number of laboratories after elimination of outliers.

n_o is the number of outliers.

$\bar{X}$ is the mean longest root length, in mm.

s_r is the standard deviation of repeatability.

CV_r is the variation coefficient of repeatability, in %.

s_R is the standard deviation of reproducibility.

CV_R is the variation coefficient of reproducibility, in %.

Table C.4 — Interlaboratory test results for the effect percentage on the mean root length

	N	n_o	$\bar{X}$	s_r	CV_r	s_R	CV_R
Lepidium sativum							
BORIC ACID	27	1	45,0	6,7	14,8	8,6	19,1
SOIL 2	7	1	38,7	4,3	11,0	9,6	24,8
Sinapis alba							
BORIC ACID	27	1	43,8	6,9	15,8	11,1	25,3
SOIL 2	7	1	43,8	6,9	15,8	8,1	18,4
WASTE SOIL	8	0	23,3	9,4	40,3	12,0	51,5
Sorghum saccharatum							
BORIC ACID	25	3	30,5	5,8	18,9	10,8	35,4
SOIL 2	7	0	34,2	14,2	41,5	17,1	50,0

N is the number of laboratories after elimination of outliers.

n_o is the number of outliers.

$\bar{X}$ is the mean effect on the mean root length, in %.

s_r is the standard deviation of repeatability.

CV_r is the variation coefficient of repeatability, in %.

s_R is the standard deviation of reproducibility.

CV_R is the variation coefficient of reproducibility, in %.

Table C.5 — Interlaboratory test results for the effect percentage on the longest root length

	N	n_o	$\bar{X}$	s_r	CV_r	s_R	CV_R
Lepidium sativum							
BORIC ACID	28	0	41,8	6,0	14,4	7,4	16,6
SOIL 2	8	0	42,9	4,2	9,7	8,4	19,7
Sinapis alba							
BORIC ACID	27	1	44,4	6,4	14,5	8,2	18,6
SOIL 2	7	1	45,1	5,2	11,4	5,7	12,8
Sorghum saccharatum							
BORIC ACID	27	1	41,2	5,3	12,9	7,5	18,1
SOIL 2	7	0	37,9	11,4	30,0	19,1	50,3

N is the number of laboratories after elimination of outliers.

n_o is the number of outliers.

$\bar{X}$ is the mean effect on the longest root length, in %.

s_r is the standard deviation of repeatability.

CV_r is the variation coefficient of repeatability, in %.

s_R is the standard deviation of reproducibility.

CV_R is the variation coefficient of reproducibility, in %.

For detailed reports on the two phytotoxicity test interlaboratory comparisons, see Reference [44].

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