TECHNICAL SPECIFICATION

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Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test

Qualité du sol — Effets des polluants vis-à-vis des champignons mycorhizogènes — Essai de germination des spores

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 10832 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Introduction

Mycorrhizal fungi are important components of the soil microbial community and key organisms in plant/soil systems. The root symbiosis they form represents a direct link between the soil and the large majority (80 %) of vascular plant species, in natural and agricultural environments. Mycorrhizal fungi provide several benefits to the host plant, including enhanced growth, improved mineral nutrition, greater drought resistance, and protection against pathogens and heavy metal stress.

Several studies have shown that mycorrhizal fungi are sensitive to pollutants such as metallic trace elements and polycyclic aromatic hydrocarbons, and to sewage sludges even when no phytotoxic effects on the host plant are observed. As mycorrhizal fungi fulfil most of the criteria for bioindicator organisms (ubiquitous in soil, sensitive to pollutants, ecologically relevant role in plant health and ecosystems), it appeared important to take them into account in hazard and environmental risk assessments linked to pollutants, contaminated soils and to the use of sewage sludge in agriculture.

Spore germination by an arbuscular mycorrhizal fungus, *Glomus mosseae*, makes up the basis of the proposed test. The first step of the symbiosis is taken into account in this test, whereas another test based on root colonization of the host plant is also under investigation.

This test can be directly performed with sludges or soils without any extraction step.

Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test

1 Scope

This Technical Specification specifies a method to evaluate the effects of pollutants on spore germination of a mycorrhizal fungus, *Glomus mosseae*. This direct acute toxicity bioassay allows the evaluation of potential effects of pollutants and contaminated soils on beneficial soil microorganisms important for plant growth within the concept of sustainable agriculture.

This Technical Specification is applicable to

— chemical substances, and

— contaminated soils, waste and soil-waste mix.

2 Normative references

The following referenced documents are indispensable for the application of this document. 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 11263, *Soil quality — Determination of phosphorus — Spectrometric determination of phosphorus soluble in sodium hydrogen carbonate solution*

ISO 11268-1, *Soil quality — Effects of pollutants on earthworms (Eisenia fetida) — Part 1: Determination of acute toxicity using artificial soil substrate*

ISO 11274, *Soil quality — Determination of the water-retention characteristic — Laboratory methods*

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

3 Terms, definitions and abbreviated terms

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

3.1
mycorrhizal fungus
ubiquitous microorganism forming symbiotic association with the roots of vascular plant species

3.2
BEG
The International Bank for the Glomeromycota

3.3
spore
asexual reproductive unit of a fungus

3.4
sporocarps
mycelium-surrounded spore group

3.5
mycelium
branched hyphae network

3.6
hyphae
filaments which compose fungus mycelium

3.7
control substrate
inert substrate, which does not affect spore germination, used as a control or dilutant

3.8
matrix
test soil, sludge or waste

3.9
sandwich
device composed of two nitrocellulose membrane filters containing the spores

NOTE The two membranes are held together with two slide frames.

3.10
trypan blue staining
non-vital staining with trypan blue used to make mycorrhizal fungus structures visible (coloured in blue)

3.11
test mixture
mixture of test substance or matrix with a control substrate

3.12
mass fraction
w_x
mass fraction of test substance or matrix inducing x % spore-germination inhibition compared to the control

4 Test methods

4.1 Principle

Spores of *Glomus mosseae* are placed between two nitrocellulose membrane filters forming a sandwich (3.9), which is placed in a Petri dish containing the test mixture (3.11) which contains the test substance or matrix (3.8) with different concentrations, diluted or not with the control substrate (3.7).

The percentage of germinated spores is estimated after 14 days.

The results are compared with a control substrate and used to estimate the 50 % spore-germination-inhibition mass fraction (w_{50}).

Determination of another w_x is also possible, but not required.

NOTE This method can be used to determine the effect of a single mass fraction.

4.2 Standard conditions

Use a growth chamber or room with a controlled temperature, (24 ± 2) °C.

Incubation is carried out in the dark (a darkroom or Petri dishes covered with aluminium foil).

5 Test materials

5.1 Distilled water

The pH of distilled water should be neutral and never less than 5,5.

5.2 Biological material

5.2.1 Fungus

Taxonomic group: Eumycota, Glomeromycota order.

Species: *Glomus mosseae* (Nicolson and Gerdeman) Gerdeman and Trappe (BEG 12).

5.2.2 Life status

Use mature spores.

5.2.3 Identification

Genbank identification number: U96139 (18s rDNA sub-unit); YO7656 (partial sequence of the 25s rDNA sub-unit) (25, 26).

5.2.4 Material

Use sporocarps (3.4) containing spores (3.3) (see Figure 1) purchased commercially[1)]. Use pot cultures that are less than five months old to extract sporocarps.

The spore-germination percentage shall be higher than 75 %. Conserve the spores and sporocarps in distilled water (5.1) at 4 °C.

Deadline before use: one week for sporocarps and two days for spores.

5.3 Control substrate

Use sand, or artificial soil in accordance with ISO 11268-1 as the control substrate (3.7).

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1) Sporocarps of the fungus *Glomus mosseae*, produced and distributed by BioRize, are an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Use sand: > 99 % silica, pH 6,6 to 7,5, particle size 0,8 mm to 1,6 mm, washed three times with distilled water (pH > 6) (5.1), then dried. The final pH shall be > 6 (see ISO 10390).

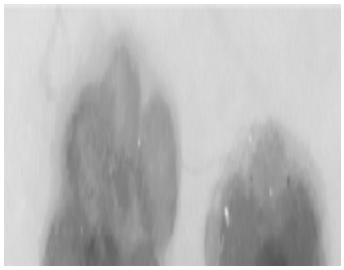
Check that spore germination in the control substrate before setting up the bioassay is > 75 %.

5.4 Reference substance

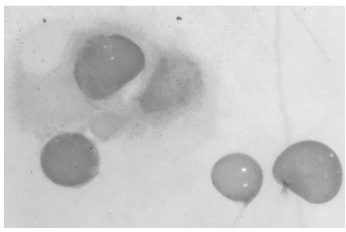
Use cadmium nitrate ($CdNO_3 \cdot 4H_2O$).

5.5 Trypan blue

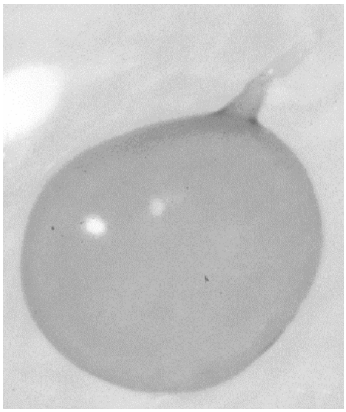
Use Trypan blue[2)], 0,5 g in 50 ml of HCl (1 %), 450 ml of H_2O, and 500 ml of glycerol.



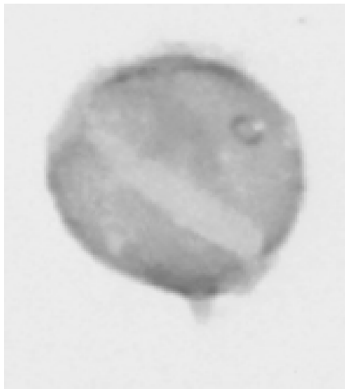
a) Sporocarps



b) Sporocarp opened



c) Intact spore



d) Broken spore

Figure 1 — Sporocarps and spores of *Glomus mosseae*

5.6 Apparatus

5.6.1 Binocular microscope, 32 × magnifications.

5.6.2 Sterile plastic Petri dishes, of diameter 9 cm.

5.6.3 Slide frames, 24 mm × 36 mm.

2) Trypan blue referenced 93595, distributed by Sigma Aldrich, is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.6.4 Nitrocellulose membrane filters, of diameter 47 mm, and porosity 0,45 µm, white, 3 mm gridlined.

5.6.5 Ultra-fine nippers/tweezers[3)].

5.6.6 Filter paper.

5.6.7 Plastic microtube, of capacity 1,5 ml.

5.6.8 Plastic film.

5.6.9 Micropipette and **cut tips**.

5.6.10 Balance, able to weigh from 0 g to 200 g, with an accuracy of 0,001 g.

5.6.11 Funnel.

6 Storage and preparation of samples

Soil samples shall be stored as specified in ISO 10381-6. Waste and sludge samples are stored at (4 ± 2) °C in tight containers. Containers used for microbiologically active sludge and waste should not be filled completely.

The following parameters should be determined for the soils, wastes and sludges to be tested:

— pH (see ISO 10390); use soil with a pH not lower than 5.5 (see Reference [18] in the Bibliography);

— water content (see ISO 11465);

— water-holding capacity (see ISO 11274);

— available phosphorus content (see ISO 11263);

— mass fraction of soluble phosphorus which shall be below 100 $mg \cdot kg^{-1}$ (see References [1], [2], [7] and [23]).

Use soils or wastes with a particle size below 4 mm in order to perform the bioassay. Otherwise, wastes should be ground and sieved to 4 mm, and soils should be sieved to 4 mm before preparing the test mixture.

7 Procedure

7.1 Biological system

7.1.1 Spore control

Glomus mosseae spores (3.3) should be yellow and bright, and have an intact and clean envelope (empty and crushed spores should be eliminated), see also Figure 1.

7.1.2 Preparation of the biological material

Number of individual spores for each concentration tested: 30 spores (3.3) per sandwich (3.9), six replicates (sandwiches) per concentration; i.e. 180 spores per concentration.

3) Ultra-thin tweezers referenced T5130 No. 5, distributed by Oxford Instruments, are an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Spores (3.3) are carefully collected under a binocular microscope (5.6.1) from the sporocarps (3.4), which are placed on a filter paper (5.6.6) that has been slightly humidified so that the spores adhere to it. Sporocarps are gently opened with ultra-fine tweezers (5.6.5) and spores can then be taken and transferred to a microtube (5.6.7) containing distilled water (5.1).

A filtration membrane (5.6.4) humidified with distilled water (5.1) is placed on humidified filter paper (5.6.6) under a binocular microscope (5.6.1). With a micropipette (5.6.9), *G. mosseae* spores (3.3), taken from the microtube (5.6.7), are placed in the middle of this membrane, inside squares which are defined by the slide frame (one spore per square defined by the gridline) (see Figure A.1). The spores are carefully covered with a second filtration membrane previously humidified, in order that the gridlines of both membranes are superposed. The two membranes are held together within two slide frames (5.6.3) to form a sandwich.

Spore sandwiches (3.9) should be quickly placed in the Petri dishes (5.6.2).

7.2 Preparation of the test mixture

7.2.1 General

The test mixture (3.11) is composed of the control substrate (3.7) and the substance or matrix (3.8) to be tested. Prepare enough test solution or mixture with the matrix.

7.2.2 Water-soluble or emulsifiable substances

For each concentration tested, dissolve or dilute the quantity of test substance required to obtain the desired concentration in distilled water (5.1).

This solution is added to a Petri dish containing 80 g of control substrate (5.3) in order to obtain 90 % of the water-holding capacity of the test mixture (3.11), see Figure 2.

Proceed in two steps as indicated in 7.3.1.

The control assay is performed as described above with distilled water (5.1).

Continue the assay as specified in 7.3.

7.2.3 Water-insoluble and organic-solvent-soluble substances

For each concentration tested, dissolve or dilute the quantity of test substance required to obtain the desired concentration in a volatile solvent (e.g. methanol).

This solution is mixed with 10 g of control substrate (5.3) (previously sampled from the quantity used to prepare the test mixture). Organic solvent is evaporated under a vacuum hood for 24 h. Transfer the test mixture (3.11) to a flask containing the rest of the test mixture and carefully homogenize.

80 g of this mixture is humidified to 90 % of the water-holding capacity with distilled water (5.1), see Figure 2.

Proceed in two steps as indicated in 7.3.2.

The control assay is performed as described above without the tested substance.

Continue the assay as specified in 7.3.

7.2.4 Non-water- or organic-solvent-soluble substances

For each concentration tested, mix the quantity of test substance required to obtain the desired concentration with the control substrate (5.3).

80 g of this mixture is humidified to 90 % of the water-holding capacity with distilled water (5.1), see Figure 2.

The control assay is performed as described above without the tested substance.

Continue the assay as specified in 7.3.

7.2.5 Solid matrices

Increasing dilution of the tested matrices is prepared with the control substrate (5.3). This test mixture (3.11) is used in two parts. Proceed in two steps as indicated in 7.3.4. Prepare enough volume for one Petri dish.

The control assay is performed without the matrix.

Continue the assay as specified in 7.3.

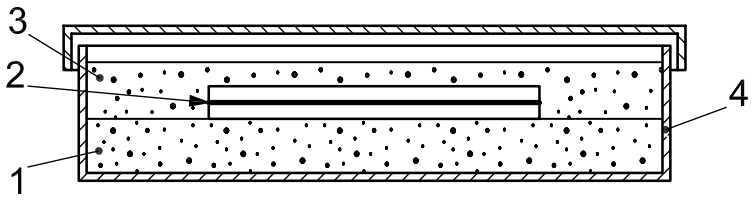
7.3 Setting up of the test

The sandwich (3.9) is transferred to a Petri dish between two layers of test mixture (3.11) (see Figure A.1).

Petri dishes, solutions and control substrate (3.7) should be prepared before the preparation of the biological material.

7.3.1 For a test mixture containing a water-soluble or emulsifiable substance

Place a first layer (about 40 g) of the control substrate (3.7) in the Petri dish. Humidify the control substrate with a fraction of the tested substance or distilled water (5.1) (control) (cf. 7.2.2). Lay the spore sandwich (3.9) on top and cover it with a second layer (about 40 g) of the same control substrate (3.7). Humidify with the remaining tested substance or distilled water (5.1) (control) in order to obtain 90 % of the water-holding capacity (see 7.2.2 and Figure 2).



Key

1 1st layer of substrate or test mixture
2 sandwich of spores
3 2nd layer of substrate or test mixture
4 Petri plate

Figure 2 — Procedure for water-soluble or emulsifiable substances

7.3.2 For a test mixture containing an organic-solvent-soluble substance

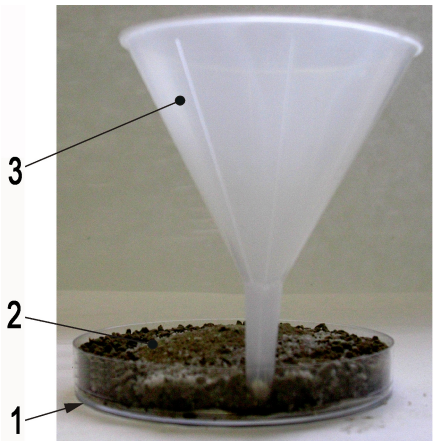
Place a first layer (about 40 g) of the test mixture (3.11) in the Petri dish. Lay the spore sandwich (3.9) on top and cover it with a second layer (about 40 g) of the same test mixture. Humidify with distilled water (5.1) in order to obtain 90 % of the water-holding capacity (see 7.2.3 and Figure 2).

7.3.3 For a test mixture containing water or an organic-solvent-insoluble substance

Place a first layer (about 40 g) of the test mixture (3.11) in the Petri dish. Lay the spore sandwich (3.9) on top and cover it with a second layer (about 40 g) of the same test mixture. Humidify with distilled water (5.1) in order to obtain 90 % of the water-holding capacity (see 7.2.4 and Figure 2).

7.3.4 For a test mixture containing a solid matrix

Place a first layer (40 g) of the test mixture (3.11) in a Petri dish, the bottom of which is lined by a moistened filter paper. The sandwich (3.9) of spores (3.3) is laid in the Petri dish and covered with a second layer (40 g) of the same test mixture. Substrates (3.7) of assay are humidified to 90 % of their water retention capacity by capillarity with distilled water (5.1). The volume of water necessary to reach 90 % of the water-retention capacity can be supplied by a funnel (5.6.11) (of which the extremity is obstructed by moist cotton wool to limit the speed of the water flowing) or a pipette in contact with the filter paper at the base of the Petri dish; see Figure 3. The Petri dish, solutions and quantities of test mixture shall be prepared before the biological system (7.1).



Key

1 Petri dish lined by a moistened filter paper
2 substrate or test mixture containing the spore sandwich
3 funnel to supply the required water quantity

Figure 3 — Procedure for solid matrices and test mixtures

7.4 Choice of the tested mass fraction

7.4.1 Preliminary assay

Choose five mass fractions of the substance or mixture and one control (e.g. 0 $mg \cdot kg^{-1}$, 0,1 $mg \cdot kg^{-1}$, 0,2 $mg \cdot kg^{-1}$, 0,5 $mg \cdot kg^{-1}$, 1 $mg \cdot kg^{-1}$ and 2 $mg \cdot kg^{-1}$ of control substrate) with 30 spores per concentration and per Petri dish. For the preliminary assay, only one concentration can be tested.

7.4.2 Final assay

Choose a range of at least five concentrations of the tested substance, mixture or matrix with a geometric progression, in order to cover concentrations which had no effect on germination and concentrations which totally inhibited it in a preliminary test. Preferably, the ratio of the geometric progression should not exceed 2.

7.5 Culture condition

The Petri dishes are sealed with plastic film (5.6.8) and incubated for 14 days at (24 ± 2) °C in the dark.

7.6 End of the assay

After 14 days, the sandwiches (3.9) containing spores are taken carefully out of the Petri dishes, always kept horizontal, rinsed with distilled water (5.1), if necessary, and submerged in a 0,05 % trypan blue (5.5) solution

for 15 min to make hyphal germination (see 3.6) visible. The sandwiches (3.9) are carefully rinsed and dried with absorbent paper and opened. The total number of spores recovered and the number of germinated spores (3.3) are counted under a binocular microscope (5.6.1).

With soils, sandwiches should be rinsed with distilled water before staining.

Hyphal (see 3.6) length after germination should be at least greater than five times the spore diameter to be counted.

8 Calculation and expression of results

8.1 Calculation

For each treatment, concentration and replicate, the number of recovered and germinated spores (3.3) is estimated. The percentage of germinated spores versus the number of recovered spores is calculated. The average percentage of germination and the standard deviation for each treatment and concentration are calculated and compared to the control.

An example of a table is given in Annex C.

Estimate the mass fraction (w_{50}) (3.12) when the dose-effect of pollutants is tested and estimate the 95 % confidence interval.

For a statistical analysis, the percentage data should be transformed to arc sine before the test.

NOTE Another value of w_x could be calculated with the appropriate method.

8.2 Expression of results

For each treatment, the germination percentage versus mass fraction is plotted on a graph.

9 Validity criteria

The results are considered as valid if

— the average number of recovered spores is greater than or equal to 25 at the end of the assay, and

— the average percentage of spore germination for the control is greater than or equal to 75 % at the end of the assay.

For information, in a ring test (n = 4) performed with cadmium nitrate, the value of w_{50} in sand was between 0,15 mg·kg^{-1} and 1,7 mg·kg^{-1}.

10 Test report

The test report shall include the following information:

a) a reference to this Technical Specification;

b) date of the assay, end of the assay and environmental conditions of the assay;

c) required data for the identification of the substance, for the preparation or the tested matrix (3.8) [origin, matrix composition (if possible), pollutants (if identified), pH, water-soluble phosphorus (P), water-holding capacity and humidity rate];

d) possible pretreatment of the tested waste or soil;

e) reference number of the used sporocarp (3.4) set, date of reception, storage duration and conditions for sporocarps and spores (3.3) before the assay;

f) reference of sand used as control substrate (3.7) (trade mark, pH, particle size);

g) natural soil characteristics if the soil is used as a control substrate (3.7);

h) preparation of the test mixture (3.11) (solvent used for organic-solvent-soluble substance);

i) table of results (Table B.1) containing, for each treatment and concentration:

   — the number of recovered and germinated spores (3.3);

   — the germination percentage of the spores (3.3);

   — the mean and the standard deviation;

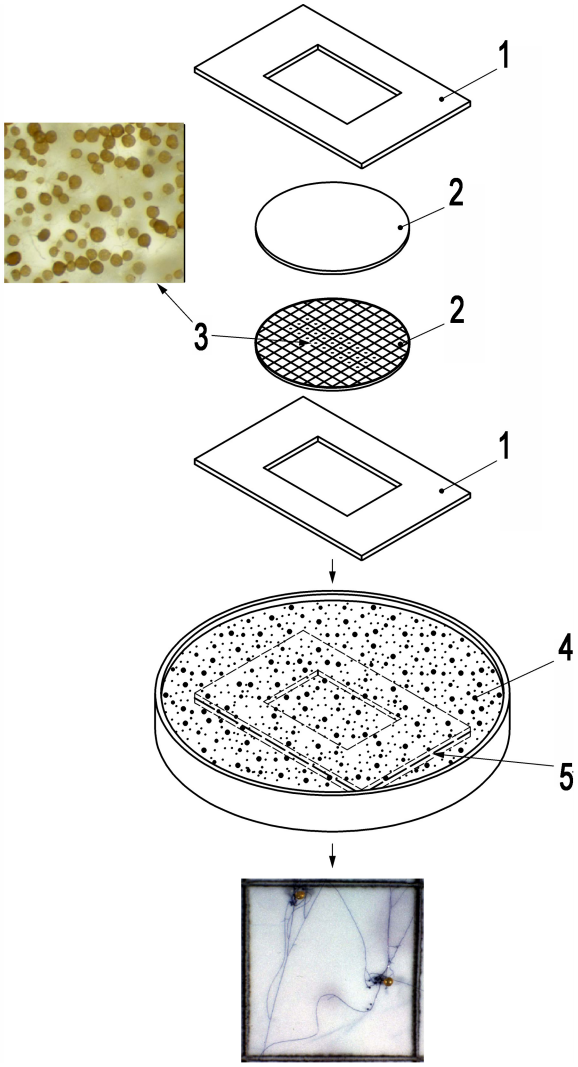
   — the graph showing data (germination percentage versus concentration);

j) any practical details not described in this Technical Specification or any incidents that may have affected the results.

Annex A
(informative)

Experimental device

Figure A.1 shows the different parts of the test apparatus and how to set it up.



Key

1 slide frame
2 filtering membrane with gridline
3 spores
4 substrate test
5 "sandwich"

Figure A.1 — Experimental device

Annex B
(informative)

Example of a table of results

Present the results within a table, such as Table B.1, with the number of replicates, number of recovered spores and germination rate.

Table B.1 — Example of a table of results

Assay (dates of start and end of assay): Tested substance:

Mass fraction mg/kg	**Replicate**	**Number of spores recovered**	**Number of germinated spores**	**Germination rate** %
	1			
	2			
	3			
	4			
	5			
	6			
			Mean	
			Standard deviation	
			Percent inhibition	
	1			
	2			
	3			
	4			
	5			
	6			
			Mean	
			Standard deviation	
			Percent inhibition	
	1			
	2			
	3			
	4			
	5			
	6			
			Mean	
			Standard deviation	
			Percent inhibition	
	1			
	2			
	3			
	4			
	5			
	6			
			Mean	
			Standard deviation	
			Percent inhibition	

Annex C
(informative)

Example of results

Within an interlaboratory comparison on spore germination with increasing mass fractions of cadmium (cadmium nitrate) and benlate (n = 4), w_{50} was between 0,15 mg·kg^{-1} and 1,7 mg·kg^{-1} for cadmium (Cd) and between 2 mg·kg^{-1} and 10 mg·kg^{-1} for benlate. An example of results obtained with Cd is given in Table C.1.

Table C.1 — Example of a table of results on spore germination with increasing mass fractions of Cd

Mass fraction of Cd mg/kg	**Replicate**	**Number of spores recovered**	**Number of germinated spores**	**Germination rate** %
Control	1	26	25	96,2
	2	28	26	92,9
	3	31	24	77,4
	4	26	22	84,6
	5	30	25	83,3
	6	27	21	77,8
			Mean	85,4
			Standard deviation	7,7
			Percent inhibition	/
0,1	1	28	15	53,6
	2	30	17	56,7
	3	30	17	56,7
	4	29	19	65,5
	5	31	19	61,3
	6	30	19	63,3
			Mean	59,5
			Standard deviation	4,6
			Percent inhibition	30,3
0,2	1	27	17	63,0
	2	22	17	77,3
	3	28	18	64,3
	4	27	18	66,7
	5	28	20	71,4
	6	30	21	70,0
			Mean	68,8
			Standard deviation	5,3
			Percent inhibition	19,4

Table C.1 (*continued*)

Mass fraction of Cd mg/kg	**Replicate**	**Number of spores recovered**	**Number of germinated spores**	**Germination rate** %
0,5	1	27	14	51,9
	2	27	14	51,9
	3	28	12	42,9
	4	28	12	42,9
	5	30	14	46,7
	6	25	13	52,0
			Mean	48,0
			Standard deviation	4,5
			Percent inhibition	77,8
1	1	29	14	48,3
	2	30	14	46,7
	3	30	16	53,3
	4	28	15	53,6
	5	24	10	41,7
	6	27	14	51,9
			Mean	49,2
			Standard deviation	4,6
			Percent inhibition	73,4
2	1	26	6	23,1
	2	27	9	33,3
	3	29	6	20,7
	4	26	8	30,8
	5	30	12	40,0
	6	30	14	46,7
			Mean	32,4
			Standard deviation	9,9

Annex D
(informative)

Precision data

An international interlaboratory comparison on the spore germination in soil was performed by seven volunteer laboratories in spring 2006. The bioassay was performed according to AFNOR XP X 31-205-1 .The test was performed with seven samples: two control substrates (sand and artificial soil), an agricultural soil, and two contaminated soils: one soil contaminated with heavy metals, and one with a multipollution (polycyclic aromatic hydrocarbons, heavy metals) contaminant. The latter one was used undiluted, diluted 1:2 and diluted 1:10 with sand. Dried soils and sporocarps were sent to the different laboratories, so that each of them used the same source of material. The soils were humidified for two weeks before use in the germination test (to equilibrate trace elements or other pollutants in the soil solution).

The laboratories received around 600 g of each sample to perform six replicates with 80 g of soil, and 96 squared filtering nitrocellulose membranes. For sample number 2, they received 300 g (instead of 600 g) because it was very light and the Petri plates were filled with 40 g instead of 80 g. As soon as they were received, the soil samples were kept at 4 °C until use. Before use, all samples were homogenized (with a good rotary shake 10 times by hand). The water-retention capacity of each sample and the quantity of water to be added to the Petri plates were already calculated and fixed so that all the laboratories used the same values.

The seven samples included two controls (sand and artificial soil), three soils (see Table D.1) and two dilutions of one of the soils:

a) sand;

b) artificial soil;

c) Bouzule (Champenoux, France): agricultural clayey soil;

d) MetalEurope (northern part of France): cultivated soil contaminated with heavy metals by atmospheric deposition from a smelter;

e) Neuves-Maisons (north-eastern part of France): industrial (coking plant site) soil contaminated with PAH and heavy metals;

f) Neuves-Maisons diluted 1:2 [sand, same as a)];

g) Neuves-Maisons diluted 1:10 [sand, same as a)].

Previous tests had shown that pure sand was a good control substrate, but results within this interlaboratory comparison (see Table D.2) were variable, suggesting that the sand to be used should be tested before performing the test. On the contrary, with the artificial soil, results were consistent and spore germination was high in all laboratories.

Table D.1 — Soil characteristics

Soil name	Bouzule	MetalEurope	Neuves-Maisons
Texture	Silty clay loam	Silt loam	**Sandy loam**
pH	7,4	7,7	**7,1**
CEC ($cmol \cdot kg^{-1}$)	14,9	15,3	**Not determined**
Organic C ($g \cdot kg^{-1}$)	16,0	16,6	**70,2**
C/N	9,4	13,5	**25,8**
Total $CaCO_3$ ($g \cdot kg^{-1}$)	8,62	7,78	**37,7**
P (Olsen) ($mg \cdot kg^{-1}$)	117	148	**300**
Total Cd ($mg \cdot kg^{-1}$)	0,29	7,87	**2,4**
Total Zn ($mg \cdot kg^{-1}$)	148	627	**2 770**
Total Pb ($mg \cdot kg^{-1}$)	50	444	**683**
Total Cu ($mg \cdot kg^{-1}$)	35	25	**95,5**
16 PAH ($mg \cdot kg^{-1}$)	—	—	**1 219**

Two laboratories had low spore-germination percentages and the results were not validated. A preliminary test could be recommended to check spore germination in new user's laboratories.

Results with the tested soils were consistent for the other laboratories, with no inhibition for the MetalEurope soil, while the other soils inhibited spore germination. A dose effect was observed, although it was not proportional to the dilution factor. It can be noticed that the MetalEurope soil did not inhibit spore germination, while the mass fraction of cadmium extractable with NH_4NO_3 was high (0,8 mg kg^{-1}), but the mass fraction of Cd extractable with water was low (0,02 mg kg^{-1}). NH_4NO_3 extraction is considered as a good method to reflect the mass fraction of Cd in plants, but our results suggested that the spore-germination test may not react in the same way as plant uptake. Although it was not known to be contaminated, the Bouzule soil inhibited spore germination. We checked that this was not due to the high clay content, since two other soils with more than 30 % clay content did not inhibit spore germination. Other unidentified parameters (no information was available on pesticide amendments) could be responsible for the germination inhibition.

Spore quality and high germination ability, as well as humidity control, are key parameters for this bioassay.

Table D.2 — Results of an interlaboratory comparison [percentage of spore germination, mean and standard deviation (SD)]

	France		Poland		Italy		Finland		Germany		Czech Republic		Spain	
Sample	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Sand	58,9	—	2,7	3,9	11,2	4,7	49,4	12,7	0	0	0,7	1,8	94,2	1,9
Artificial soil	91,0	6,9	88,4	8,4	84,6	1,5	79,8	8,9	7,6	10,4	76,2	16,3	92,7	3,8
Bouzule	7,5	4,5	0,03	0,1	5,5	3,7	18	18,6	0	0	0	0	39,7	13,5
Métal-Europe	77	21,8	91,5	4,1	85,7	1,5	58,4	16,8	20,2	11,1	3,7	4,1	58,9	52,3
Neuves-Maisons	18,1	24,8	36	25,5	12,4	9,6	5,2	10,0	0	0	1,3	3,1	26,1	21,0
Neuves-M 1:2	60,5	19,9	27,5	15,1	22,0	6,6	15,2	23,0	11,3	24,5	3,1	3,7	89,6	10,4
Neuves-M 1:10	58,3	19,2	64,3	12,3	30,3	7,9	50,6	21,7	53,8	5,4	0	0	75,9	15,2

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