PHYTOTOXKIT

Seed germination and early growth microbiotest with higher plants

STANDARD OPERATIONAL PROCEDURE

TABLE OF CONTENTS

INTRODUCTION TO THE PHYTOTOXKIT MICROBIOTEST

ORIGIN

The conventional phytotoxicity tests prescribed by national and international organisations are complex, space demanding and time consuming, and there is a clear need for more practical and user-friendly methods to determine the impact of pollutants on higher plants.
The specific concept of the Phytotoxkit microbiotest bypasses the unpractical and laborious manipulations of the germinated plants inherent to many phytotoxicity assays and allows direct length measurements of roots and shoots in the special test containers, by image analysis .

SCOPE

The Phytotoxkit microbiotest measures the decrease (or the absence) of seed germination and of the growth of the young roots after a few days of exposure of seeds of selected higher plants to toxicants or to contaminated soils in comparison to the controls in a reference soil.
Besides toxicity assessment of contaminated soils, the Phytotoxkit is also suited for sludges, sediments, composts and effluents for irrigation, as well as for toxicity determinations and toxicity ranking of pure chemicals and biocides.

PRINCIPLE

The Phytotoxkit makes use of unique flat and shallow transparent test plates composed of two compartments, the lower one of which contains soil saturated to the water holding capacity (WHC).
Seeds of the selected test plants are positioned at equal distance near the middle ridge of the test plate on a black filter paper placed on top of the hydrated soil.
After closing the test plates with their transparent cover by means of the unique click system, the test plates are placed vertically in a holder and incubated at 25 °C (+/- 1 °C).

The length of the incubation period (minimum 3 days) depends on the time of germination of the seeds and the growth speed of the roots, which are “species-dependent”.
At the end of the incubation period a “digital” picture is taken of the test plates with the germinated plants (either with a digital camera, a webcam camera or a flatbed paper scanner) for storage in a computer file.
The analyses and the length measurements (by image analysis) can be made immediately or postponed to any appropriate timing.

ASSETS OF THE PHYTOTOXKIT

The Phytotoxkit has multiple advantages over conventional phytotoxicity assays :

- the set-up is simple and rapid
- the test plates are small and require little bench space or incubation space
- the transparent test plates allow for direct observation of the germinated seeds without any manipulation
- the pictures of the test plates with the germinated seeds are stored as computer files which allows to postpone the measurements
- the length measurements of the roots is rapid and automatic by the use of image analysis techniques
- multiple tests can be set up concurrently
- the test procedure is highly standardized and its precision has been evaluated in an "International Interlaboratory Comparison" in which 28 laboratories from 12 countries have participated
- Validity criteria have been selected for the assay and a methodology has been worked out for a reference test (quality control test) with boric acid.

FEATURES

The Phytotoxkit microbiotest is very flexible and can be applied with any type of plant seed and test soil, in comparison to a control soil.

Three plant species have been selected for the standard Phytotoxkit microbiotest, on the basis of the rapid germination of the seeds and the growth of the roots which allows to complete the assays after only 3 days of incubation :

1) the monocotyl Sorgho (*Sorghum saccharatum*)
2) the dicotyl garden cress (*Lepidium sativum*)
3) the dicotyl mustard (*Sinapis alba*).

The standard Phytotoxkit microbiotest analyses two kinds of effects in the test soil in comparison to a control soil: the decrease of seed germination and the decrease of root growth (in analogy to ISO standard 11269-1 "Determination of the effects of pollutants on soil flora – Part 1. Method for the measurement of inhibition of root growth").

Other endpoints can, however, also be analysed, such as e.g. shoot growth and morphological characteristics of the early plant stages.

The assays are carried out in 3 replicates with each of the 3 test plants.

A special artificial reference soil has been developed for the Phytotoxkit microbiotest. The control soil is analogous to the artificial soil recommended by OECD for soil toxicity tests with earthworms, and by ISO for toxicity tests with plants. The control soil is composed of sand, kaolin, peat and adjusted for pH with calcium carbonate.

N.B. : Since the amount of organic matter (peat) in the original OECD formula is not representative (i.e. too high) for most natural soils, the proportions of the 3 components have been adjusted for the Phytotoxkit reference soil to 85% sand, 10% kaolin and 5% peat. The latter composition reflects very well the composition of the LUFA 2.2. "natural soil", which is a standard soil used extensively as a reference soil.

The only equipment needed for performance of the tests is an incubator or a temperature controlled room at 25 °C (+/- 1 °C), and a digital camera, a webcam camera or a flatbed paper scanner to shoot pictures of the test plates with the germinated seeds, for storage in a computer file.

The length measurements of the germinated seeds can be made with any suitable image analysis programme such as e.g. "ImageJ" which can be downloaded directly from the Internet, or can be provided at no charge.

SHELF LIFE

The shelf life of the Phytotoxkit is function of the germination potential and the growth strength (vigour) of the seeds. If the seeds are stored properly, the shelf life is at least half a year.

SENSITIVITY

The Phytotoxkit microbiotest (with the 3 test species indicated above) has been compared with conventional phytotoxicity test methods for pure chemicals, contaminated soils and composts, and the effects were similar to those obtained with the conventional assays.

CONTENTS OF THE PHYTOTOXKIT

Test plates
18 transparent test plates (21 x 15.5 x 0.8 cm) in polyvinylchloride (PVC). The test plates are composed of a bottom part separated by a middle ridge into two compartments, and a flat cover. Both parts have small rectangular cavities on their side for closing the plates tightly by a unique click system. The test plates are provided with marks for the exact positioning of the seeds and with a label to record the specifics of each test plate (type of soil, type of seed, number of the replicate).

Envelope with filter papers and labels
An envelope containing 18 rectangular high purity black seed testing filter papers (8.7 x 12.5 cm, thickness 0.54 mm) with rounded edges, to be placed on top of the soil in the bottom compartment of the test plates, and 18 labels for identification of the individual test plates.

Test plate holders
3 cardboard holders (17 x 6 x 10.5 cm) for vertical incubation of 6 test plates each.

Bags with reference soil
9 plastic bags with zipper, each containing 90 cm^3 reference soil (artificial OECD soil) for the 3 control tests on each plant species.

Tubes with seeds
3 tubes with quality controlled seeds of the selected plant species : the monocotyl *Sorghum saccharatum* (Sorgho) and the dicotyls *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard).
For optimal germination and longest shelf life the tubes with seeds should be stored in the refrigerator (5 °C ($\pm$ 2 °C)).

Microsieve cylinder
A small plastic cylinder provided at the bottom with a nylon gauze, to be used for determination of the water holding capacity (WHC) of the test soils.

Wide mouth micropipette
A plastic micropipette to be used with the microsieve cylinder for determination of the water holding capacity of the test soils.

Results sheets
3 sheets for the scoring of the mean germination data and the mean root lengths for each type of seed in the control soil and the test soil, and for calculation of the inhibition percentages.

Standard Operational Procedure manual
A detailed brochure with all the instructions for the performance of the standard Phytotoxkit microbiotest.

Bench protocol
An abbreviated version of the Standard Operational Procedure manual.

Specification sheet
A sheet indicating the batch number and the shelf life of the seeds and the batch number of the reference soil.

PHYTOTOXKIT ACCESSORIES PACKAGE

The Phytotoxkit accessories package contains a number of specific materials for easy test preparation. This package is not included in the Phytotoxkit and has to be ordered separately. It can, however, be used continuously.
The accessories package is composed of the following items (see Figure 1) :

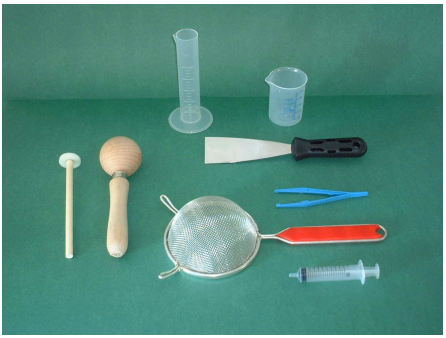


Figure 1 : Phytotoxkit accessories package

Beaker
A 100 ml graduated polypropylene beaker, for measurement of the 90 cm^3 soil to be put into the test plates and for determination of the water saturation volume of the test soils.

Graduated cylinder
A 50 ml graduated polypropylene cylinder to be used for determination of the water saturation volume of the test soils.

Syringe
A 50 ml synthetic syringe, for hydration of the soil samples in the test plates.

Sieve
A round (12 cm diameter) metal sieve of 2 mm mesh for sieving the test soils prior to use for the tests.

Spherical pestle
A spherical (5.5 cm diameter) wooden pestle to facilitate sieving of the test soil.

Flat pestle
A small (3 cm diameter) flat pestle to compact sieved test soil in the beaker.

Spatula
A spatula with a 4 cm blade in stainless steel, to spread the hydrated soil samples evenly in the lower compartment of the test plates and to flatten the surface of the soil.

Tweezers
A pair of plastic tweezers for easy transfer and positioning of the seeds on the paper filter in the test containers.

Flat bottom pestle
A small pestle with flat bottom for flattening the soils in test plates.

TEST PROCEDURE

*The test procedure described hereafter in detail has been visualised in a "**slide show**" with photos and text showing step by step the manipulations involved in the performance of the assay. This slide show is accessible on the website www.microbiotests.be in the section "Technical Info - Slide Shows - Phytotoxkit Slide Show".*

1. QUICK METHOD FOR DETERMINATION OF THE WATER HOLDING CAPACITY (WHC) OF THE TEST SOILS

The assays with the standard Phytotoxkit microbiotest are carried out at "water saturation" of the soils at the start of the tests.

A simple and quick procedure has been worked out specifically for the Phytotoxkit to determine the volume of water to be added to air-dried soils in the test plates to reach the WHC.

1. Control soil

The WHC of the reference soil has been determined experimentally. The ratio water/soil (on a vol/vol basis) was found to be 0.39 which means that 35 ml distilled or deionized water must be added to the (90 cm^3) control soil in the test plate to obtain 100% saturation.

Each Phytotoxkit contains 9 bags with exactly 90 cm^3 dry reference soil (for the 3 replicate tests with each type of seeds) to be poured in the bottom compartment of the test plates, prior to hydration of the soil.

2. Test soil

A. Air-drying of test soil

1. Take about 1 dm^3 test soil and spread the soil in a thin layer on a flat surface.
2. Let the soil dry until it 'pulverises' easily on pressing between thumb and finger.

B. Determination of the water holding capacity (see Figure 2)

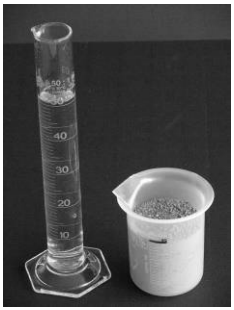
1 Sieve the dried soil through a sieve with a 2 mm mesh to eliminate all coarse material.
2. Fill a 50 ml graduated cylinder with distilled water to the mark and fill a graduated 100 ml beaker to the 90 ml mark with dry soil.
3. Slowly pour the 50 ml distilled water on top of the soil in the beaker.
4. Mix the water thoroughly with the soil with a spatula till the soil is completely water-saturated.
   Wait for the soil/water mixture to reach equilibrium, leading to a water-saturated soil phase with a layer of water on top.
5. Take the microsieve cylinder and bring it down vertically in the beaker with water-saturated soil, till the bottom of the sieve touches the supernatant.
   Gently lower the microsieve cylinder a little further down, so that it gradually fills with supernatant.
6. Suck up the water in the microsieve with the wide mouth micropipette.
7. Transfer the retrieved water into the graduated cylinder.
8. Repeat the former operations, pushing each time the microsieve cylinder a little further down into the beaker, until no water penetrates anymore in the microsieve.
9. Calculate the volume of water (Vsat) needed for complete hydration of the test soil.
   This volume is equivalent to the volume of water that has been added (= 50 ml) minus the volume of supernatant water (S) recovered in the graduated cylinder (Vsat = 50 - S).
10. Discard the wet soil from the beaker and clean and dry the beaker thoroughly for further use.

2. ADDITION OF REFERENCE SOIL AND TEST SOIL TO THE TEST PLATES AND HYDRATION OF THE SOILS

1. Control soil (see Figure 3)

1. Take one bag with (90 cm^3) reference soil and pour the contents in the lower compartment of a test plate.
2. Take a 50 ml syringe and fill it to the 35 ml mark with distilled water.
3. Hold the syringe vertically above the soil in the test plate and empty the contents by dropping the water slowly over the whole surface of the soil in the test plate.

FIGURE 2 : DETERMINATION OF THE WATER HOLDING CAPACITY (WHC) OF THE TEST SOIL



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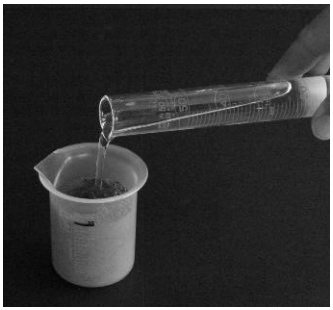

Step 3

Step 4

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Step 6

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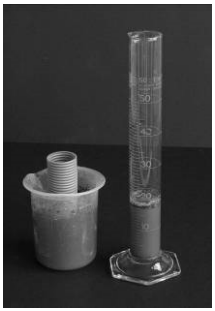
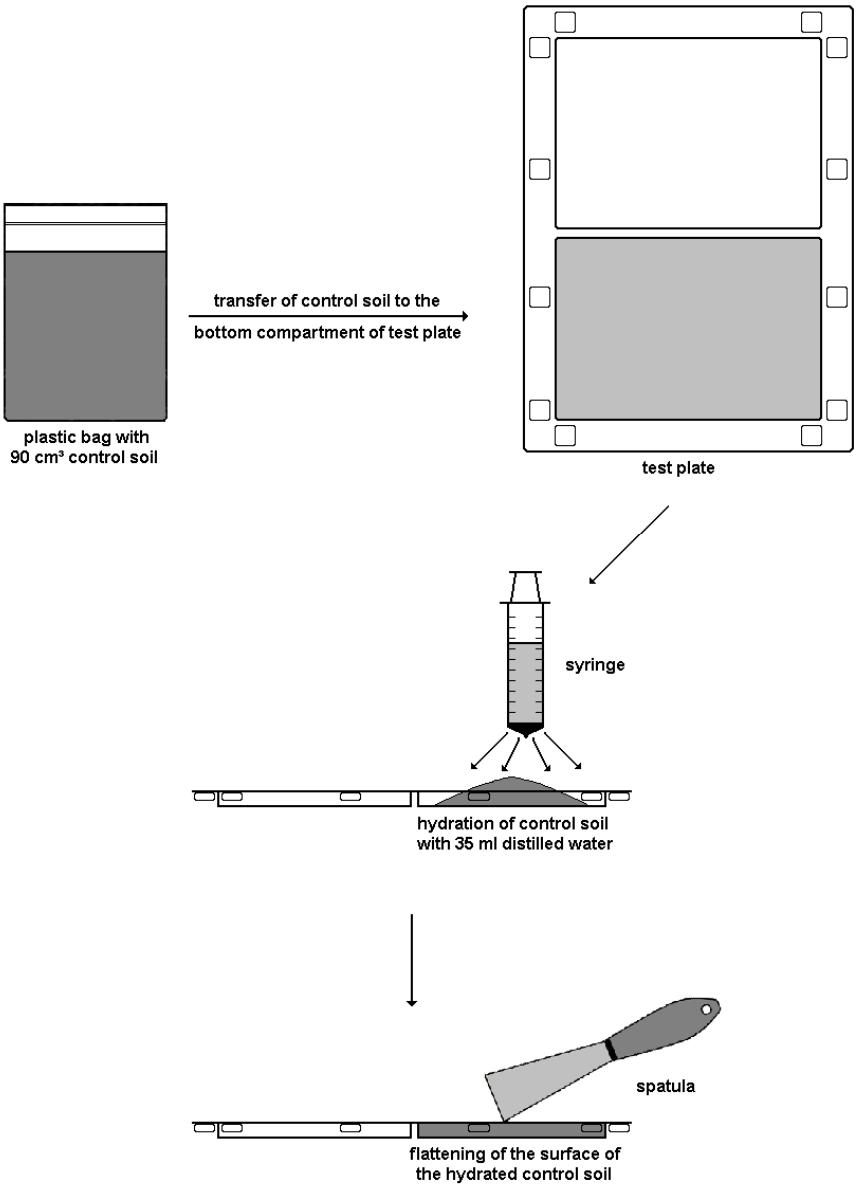


Step 8

Vsat = (50 - S)ml

Step 9

FIGURE 3 : ADDITION OF CONTROL SOIL TO TEST PLATE AND HYDRATION



4. Wait a few moments for the water to hydrate the reference soil totally.
5. With the aid of a spatula, spread the wet soil evenly over the total surface of the bottom compartment of the test plate and flatten the surface of the soil to obtain a layer of uniform depth.

N.B. It is imperative that the surface of the wet soil is totally flat to ensure a uniform root growth of all the germinated seeds.

6. Repeat the former operations for the 8 other control test plates.

2. Test soil (see Figure 4)

1. Fill the 100 ml beaker to the 90 ml mark with the sieved test soil.
2. Transfer this volume of soil into the bottom compartment of a test plate.
3. Hydrate the test soil as indicated above for the reference soil by spreading a volume of distilled water equal to Vsat over the entire surface of the soil in the test plate.
4. Distribute the wet soil evenly with a spatula over the bottom compartment of the test plate and flatten the surface of the soil.

N.B. Instead of measuring repeatedly an exact volume of 90 cm^3 of soil in a beaker, one can also determine the corresponding weight of test soil, and prepare such weights for transfer into the corresponding test plates.

5. Repeat the former operations for the 8 other plates with test soil.

3. Alternative procedure for the surface flattening of the soil in the test plates (see Figure 5)

The transfer of soil in the bottom compartment of the test plate, followed by the flattening of the surface of the hydrated soil is quite a time consuming operation, since one has to make sure that the entire surface of the soil in the test plate is totally flat. Furthermore, the flattening operation also regularly leads to “spilling” of soil over the rims of the test plate.

These two drawbacks can be solved with the aid of a rectangular plastic strip of 2 cm height, and a small flat-bottom pestle with a grip.

FIGURE 4 : ADDITION OF TEST SOIL TO TEST PLATE AND HYDRATION

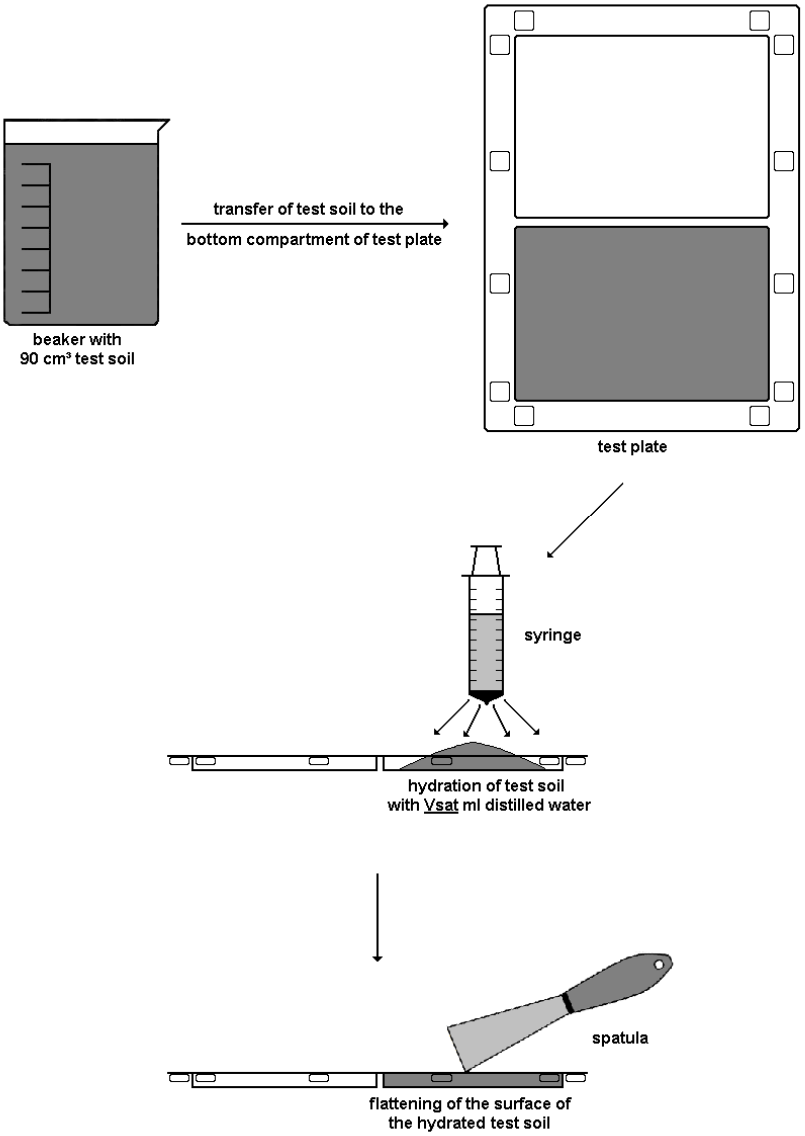
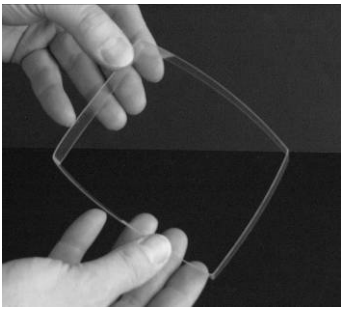
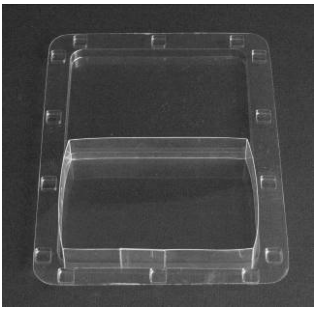


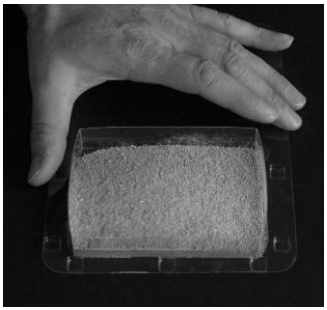
FIGURE 5 : ALTERNATIVE PROCEDURE FOR THE SURFACE FLATTENING OF THE SOIL IN THE TEST PLATES





Step 1

Step 2



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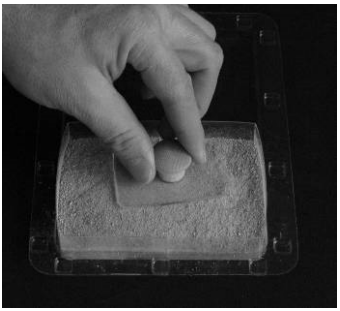

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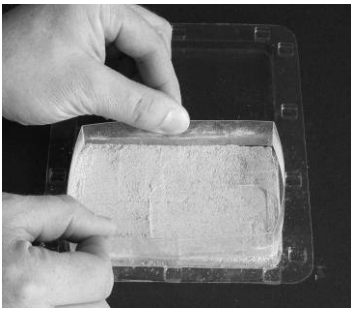


Step 5

N.B. : These two items are not included in the Phytotoxkit, but a package of 20 rectangular plastic strips and one flat-bottom pestle are part of the Phytotoxkit accessories package. The rectangular plastic strips and the pestle can also be ordered separately.

Procedure

1. Take a rectangular plastic strip and put it in the bottom compartment of the test plate.
2. Pour the contents of one bag of reference soil (or the contents of the beaker with 90 cm^3 of test soil*) into the bottom part of the test plate.
3. Place the test plate on a flat surface and shake gently to distribute the soil evenly over the whole surface inside the rectangular strip.
4. Flatten the surface of the soil with the aid of the flat-bottom pestle.
5. Remove the rectangular plastic strip by lifting it up carefully.

Proceed further with the hydration of the soil as indicated in Section 1 or 2.

** N.B. : This alternative procedure cannot be applied on soils which have a high water content.*

3. PLACING OF THE FILTER PAPER AND THE SEEDS, AND CLOSING OF THE TEST PLATES (see Figure 6)

1. Placing of the filter paper

Put one black filter paper on top of the hydrated (control and test) soils in all the test plates and wait until the filter is completely wet.

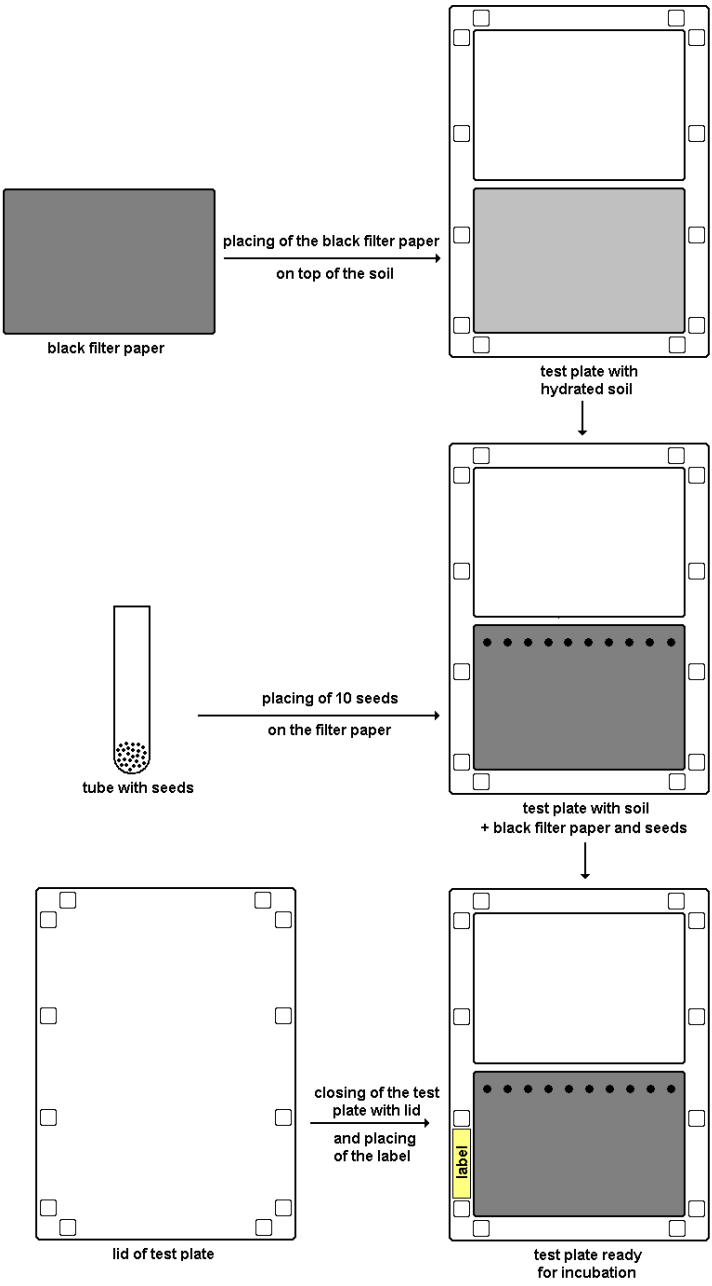
N.B. If air bubbles are trapped under the filter paper, lift the paper up and put it back slowly.

2. Placing of the seeds and closing of the test plates

N.B. The tests are carried out in 3 replicates with 3 different types of seeds, hence 6 test plates have to be inoculated with each type of seed (i.e. 3 plates with the test soil and 3 with the control soil).

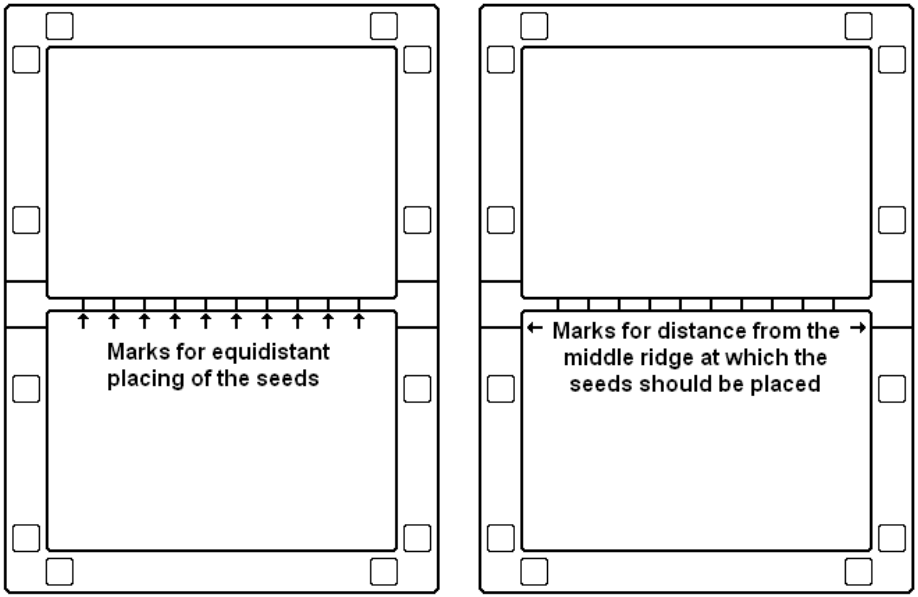
1. Place 10 seeds of the same plant on top of the filter paper in one row and at equal distance of each other. The seeds shall be placed near the top of the filter paper, at about 1 cm of the middle ridge of the test plate.

FIGURE 6 : PLACING OF THE FILTER PAPER AND THE SEEDS IN THE TEST PLATE



The correct placing of the 10 seeds is facilitated by the 10 marks at equidistant intervals on the middle ridge, and the mark on the two lateral sides of the test plate (see Figure 7).

FIGURE 7 : TEST PLATE SHOWING THE MARKS FOR CORRECT PLACING OF THE SEEDS



2. Carefully place the cover of the test plate on the bottom part and click the protruding parts of the cover into the corresponding holes of the bottom, to close the test plate tightly.

N.B. This "closing" operation shall be started in the middle of the test plate, in order to avoid that the position of the seeds changes during the closing operation.

3. Write the specifics of the test plate (type of soil, type of seed, number of the replicate) on one of the small labels (use a waterproof pen !) and stick this label vertically on the left outer edge of the bottom compartment of the test plate, in between the rectangular cavities.
4. Repeat this operation for each seed for the 3 control test plates and the 3 replicates with test soil.

4. INCUBATION OF THE TEST PLATES (see Figure 8)

1. Put the 6 test plates inoculated with the same type of seed (3 with test soil and 3 with control soil) vertically in one of the cardboard holders.
2. Repeat this operation for the 2 other seeds.
3. Put the cardboard holders with the test plates in the incubator and **incubate at 25 °C (+/- 1 °C) for 3 days.**

N.B. Illumination does not seem to play a significant role for the germination of the seeds nor the growth of the roots during the short incubation time. For uniformity reasons it is, however, advised not to provide illumination in the incubator during the test period.

5. IMAGE RECORDING AT THE END OF THE EXPOSURE PERIOD

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.

1. Image recording with a webcam camera

Follow the specific instructions of the webcam camera to take a computer photo of the test plate, which then should be saved as a JPEG file.

2. Image recording with a digital camera

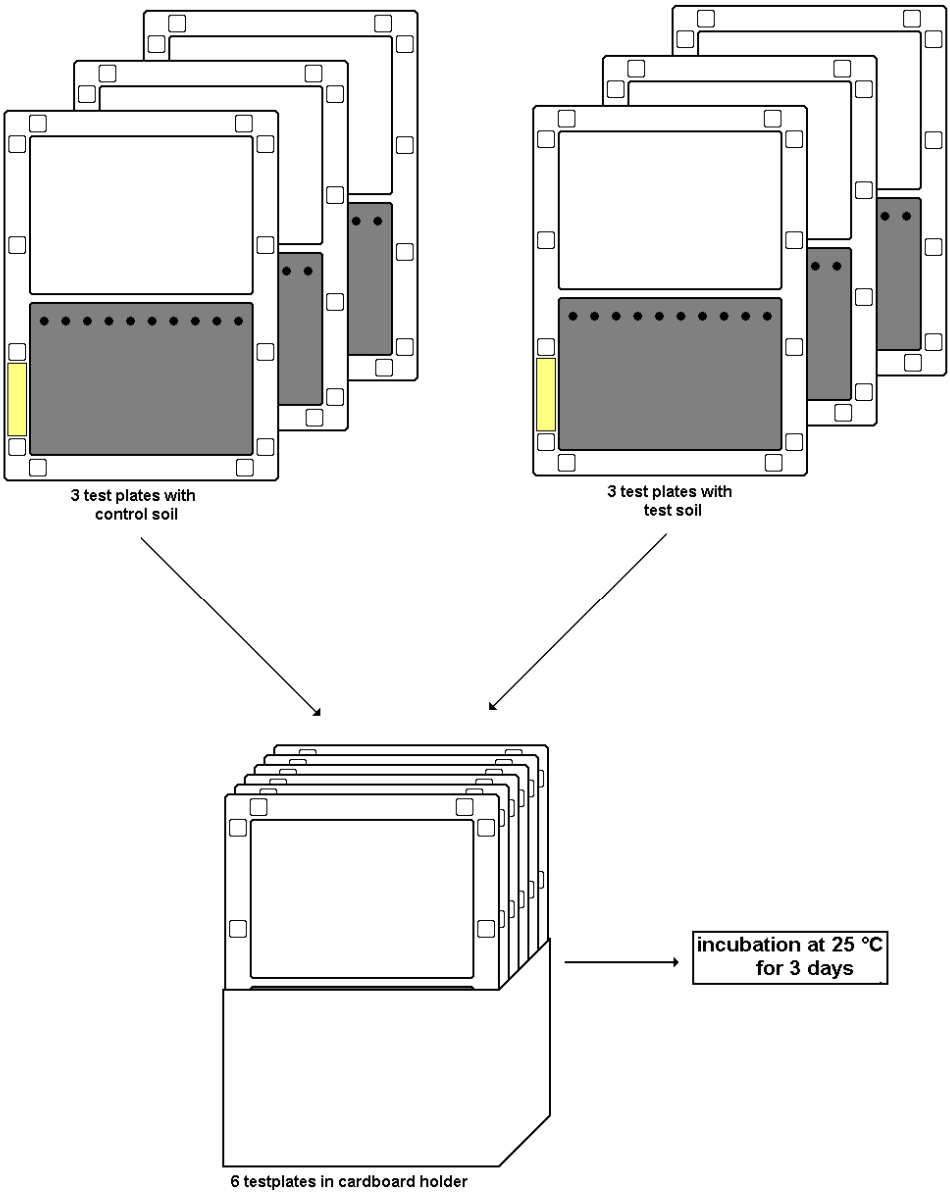
The digital camera can be mounted on a vertical stand or a stand with a telescopic arm.

Depending on the type of stand, the test plates shall be placed “horizontally” or “vertically” to take the photo.

In both cases the distance between the camera and the test plate must be selected such that the total surface of the bottom compartment of the test plate fills the screen of the LCD monitor (or the viewfinder), including the label on the left side.

After shooting the pictures, the frames shall be transferred to the computer and stored as JPEG files in the selected Directory.

FIGURE 8 : INCUBATION OF THE TEST PLATES



3. Image recording with a flatbed paper scanner

1. Put the test plate "surface down" on the paper scanner.
2. Record the image of the test plate with the appropriate programme.
3. Give the file a name (with a JPEG extension) and store it in the selected Directory.

N.B. It happens that condensation occurs on the inside of the lid of the test plates which interferes with the visibility of the roots. In such case, the lid shall be lifted off very carefully from the bottom part of the test plate prior to shooting the picture. During this operation, care has to be taken that the germinated plants don't stick to the cover.
The condensation has then to be wiped off from the lid and the latter placed back to shoot the picture. In case the picture is made with a webcam camera or a digital camera it is not even necessary to put the lid back on the test plate.

6. ANALYSIS AND MEASUREMENTS OF THE GERMINATED SEEDS IN THE SAVED PHOTOS OF THE TEST PLATES

A major asset of the Phytotoxkit test procedure is that contrary to most conventional phytotoxicity assays the analyses and length measurements don't need to be made immediately at the end of the exposure period, but can be postponed to any appropriate timing. The computer file with the saved images remains available for further analyses and measurements.

6.1. Counting of the number of germinated seeds

1. Open the file with the saved Image of a test plate with one of the seed species for the Reference soil, and count the number of germinated seeds.
2. Record the figure in the paper Results Sheet in Section "A. Seed germination" - Replicate 1.
3. Proceed similarly for the second and the third test plate of the same seed species and record the data in Replicate 2 and Replicate 3 in the Results Sheet.
4. Proceed similarly for the 2 test plates with Reference soil for the 2 other seed species and record the data in the appropriate section of the similar Results Sheets.

5. Proceed similarly for counting the number of germinated seeds in the test plates with Test soil and record the data in the corresponding sections in the Result Sheets.

N.B. : For reasons of uniformity only the seeds which have developed a root of at least 1 mm length shall be considered as being germinated.

6.2. Measurement of the root lengths of the germinated seeds

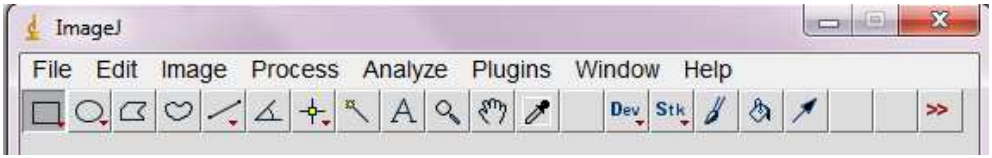
These measurements will be performed with the aid of an "Image Analysis program", such as e.g. the Image J program (which can be downloaded free of charge from the Internet).

The procedure for the root measurements with the Image J program is given in detail hereafter.

IMAGE J

Access to the picture of the test plate and increase of the picture on the screen to the size of the bottom compartment of the test plate

1. Open the Image J program - A horizontal bar appears with a number of icons and words.



2. Go to Analyze and click Set Measurements. Make sure that none of the checkboxes are ticked (untick them if they have been ticked).
   Then click OK (to save the new settings).
3. Go to File and click Open image.
4. Select the Directory and click on the file with the saved photo of the test plate.
5. Enlarge the picture of the test plate by clicking the + key several times.
6. Move the picture on the screen by pressing the space bar (and keeping it pressed) and moving "the mouse" of the computer in the desired direction.

N.B. : when keeping the space bar pressed, the + sign changes to a "small hand".

6. Enlarge the picture on the screen till the bottom compartment of the test plate almost totally fills up the computer screen (if too large, the size can be decreased with the - key).

Calibration of the measurements

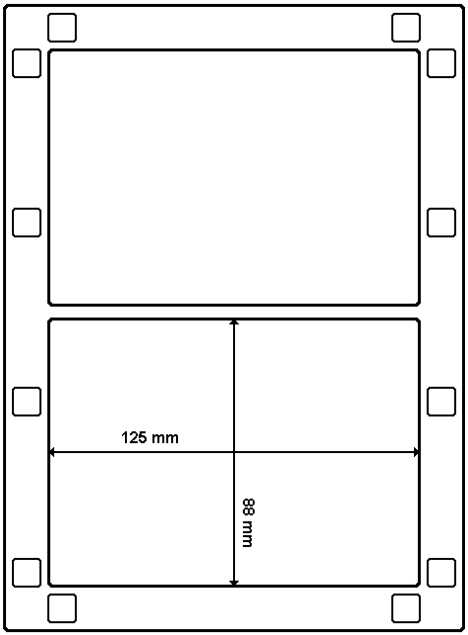
1. Click Image, then Type and 8 bit color - Click OK.
2. Click the icon / (the icon on the right of the icon showing a heart).

N.B. : if this icon is in another view (e.g. showing an indented line instead of a full line), click right on the mouse and select "straight line".

3. Position the cursor on the left border of the bottom compartment of the test plate and draw a straight line from the left border to the right border (= the width of the bottom compartment of the test plate) (see Figure 7).

N.B. : Alternatively a straight "vertical" line can also be drawn, from the top border to the bottom border.

FIGURE 7 : CALIBRATION OF LENGTH MEASUREMENTS (in millimeter)



4. Click Analyze and Set scale.
5. Fill out the box Known distance with the figure **125** (in case a horizontal line has been drawn) or **88** (in case a vertical line has been drawn).
6. Type "mm" in the box Unit of length.
7. Click OK (this will subsequently automatically make the calculation of the root lengths in mm).

Measurement of the root lengths

1. Click on the icon / and click right on the mouse to select "free hand line" (the icon will now show an indented line).
2. Move the + cursor on the screen to the place where the root of the first germinated seed protrudes from the seed and then move the cursor along the total length of the root.

N.B. : this operation has to be made "in one continuous movement". In case of a problem during this measurement, stop drawing the line and start the measurement again.

NOTE : For the monocotyl species Sorghum saccharatum, the root starts exactly at the point where it protrudes from the seed.
For the dicotyl species Lepidium sativum and Sinapis alba, the boundary between the root and the shoot is situated exactly at the point where the tiny "secondary" roots start to develop laterally on the (primary) root. This boundary is furthermore also relatively easy to find, since the shoot is somewhat thicker than the root (see Photo)

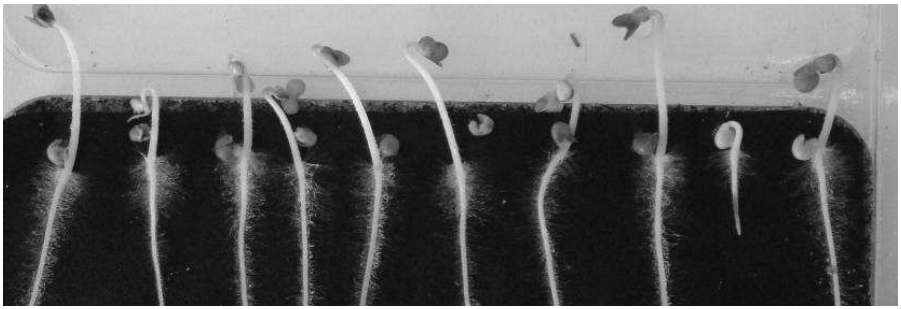


Photo of part of a test plate with germinated seeds of Sinapis alba, showing the lateral secondary roots on the (primary) roots

IMPORTANT REMARK
Similarly to what is mentioned in Section 6.1. with regard to seed germination, measurements of root lengths shall only be made on the seeds which have developed a root of at least 1 mm length.

3. Simultaneously click Ctrl and the letter M on the keyboard. This will open the Result box and show the first length measurement.
4. Shift the + cursor to the second root and proceed similarly to perform the length measurement of this root.
5. Save the result of this measurement by clicking Ctrl and M.

N.B. : as long as "Ctrl and M" are not clicked, measurement values will not be saved in the Result box.

6. Proceed further similarly for all the other roots of the test plate.

Saving of the area measurements

1. Go to the Results box (which contains the length values of all the germinated seeds in the test plate) and click on all the values (which will then show up on a black background).
2. Click right on the mouse and click Copy.
3. Open Excel and click Paste to transfer the root length data to the Excel sheet.
4. Save the Excel file and give it a name (e.g. "root lengths germinated seeds first test plate - species X - Control soil").
5. Close the Image J program for the analyzed test plate.
6. Open the Image J program again and proceed similarly as indicated above for the root length measurements of the germinated seeds in the other test plates.
7. Save each time the data for each test plate in an Excel file with a specific name.

Data treatment

1. Open the Excel sheets with the saved root length data and calculate the mean root length of all the germinated seeds for each test plate.
2. Record the root length values in the Result sheets of the corresponding sections for the Reference soil and the Test soil for each test species.

3. Calculate and record the mean root length for the 3 replicate test plates for the Reference soil (= A) and the Test soil (= B) for the 3 test species in the corresponding Result Sheets.
4. Calculate the percentage inhibition of root growth in the test soil with the formula :

$$\frac{A-B}{A} \text{ x } 100 \text{ = ... \%}$$

Measurement of the root length of only "the longest" root.

A substantial saving of time can be made by measuring only the length of "the longest" root, in each test plate, instead of measuring the length of the roots of all the germinated seeds.
Extensive research has indeed shown that the inhibition percentages calculated from the measurement data of only the longest root in each test plate are very similar to those obtained from the measurement data of all the roots in each test plate.
The figure of the longest root in each test plate shall be recorded in the Result Sheet in section B - Root growth (changing the wording "mean root length" by "length longest root").

NOTE : An Excel program for the Data Treatment of the "Phytotoxkit/ Phytotestkit microbiotest" has been worked out by the company MicroBioTests Inc.
This program allows to transfer the root length measurement data from Image J in an Excel Sheet which automatically calculates the percentage root growth inhibition.

This Excel program can be obtained free of charge from MicroBioTests Inc.

PHYTOTOXKIT

RESULTS SHEET

Date : .....

Name of operator : .....

Test soil : .....

Plant species : .....

A. SEED GERMINATION

Reference soil	Mean number of germinated seeds	Replicate 1	
		Replicate 2	
		Replicate 3	
		All replicates (= A)	

Test soil	Mean number of germinated seeds	Replicate 1	
		Replicate 2	
		Replicate 3	
		All replicates (= B)	

Percentage inhibition of seed germination in test soil :

$$\frac{A-B}{A} \times 100 = \quad \%$$

B. ROOT GROWTH

Reference soil	Mean root length (in mm)	Replicate 1	
		Replicate 2	
		Replicate 3	
		All replicates (= A)	

Test soil	Mean root length (in mm)	Replicate 1	
		Replicate 2	
		Replicate 3	
		All replicates (= B)	

Percentage inhibition of root growth in test soil :

$$\frac{A-B}{A} \times 100 = \quad \%$$

7. VALIDITY CRITERIA OF THE PHYTOTOXKIT MICROBIOTEST

From the extensive set of data generated in an "International Interlaboratory Comparison on the Phytotoxkit" (see Section 10 hereunder) the following 2 validity criteria for Phytotoxkit assays have been selected :

1. The mean germination success in the control test plates must be at least 70% for each of the 3 plant test species.
2. The minimum mean length of the roots in the control test plates must be at least 30 mm for each of the 3 plant test species.

N.B. : in case only the longest root in each test plate is measured, the minimum mean length in the control test plates must be at least 40 mm for each of the 3 plant test species.

8. REFERENCE TEST

Subsequent to extensive preliminary research in the company MicroBioTests Inc. an "International Interlaboratory Comparison on the Phytotoxkit" has been organised under the coordination of Dr. R. Baudo of the C.N.R. Istituto per lo Studio degli Ecosystemi in Pallanza, Italy.
Twenty eight laboratories from 12 countries participated in this ringtest, each performing a test with the 3 plant species on one concentration of the selected reference chemical boric acid.

From the outcome of the International Interlaboratory Comparison on the Phytotoxkit (which generated more than 10.000 data) two validity criteria for Phytotoxkit assays have been selected (see Section 9 above) and a procedure for a reference test with one concentration of boric acid (250 mg/kg control soil) has been worked out which is described hereunder.

Test procedure for a reference test with one concentration of boric acid (250 mg/kg control soil)

Test plates with control soil are spiked with a solution of boric acid, in order to obtain a concentration of 250 mg boric acid per kg control soil, after hydrating the soil in the test plates with 35 ml boric acid solution. The assay is performed with the 3 plant test species in parallel to test plates hydrated with distilled water, and in 3 replicates.

1. Weigh 714 mg boric acid on an analytical balance and transfer it into a 1 litre volumetric flask.

2. Add distilled water to the 1 litre mark, cap the flask and shake to dissolve the boric acid and obtain a uniform concentration.

3. Take 18 bags of (90 ml) reference soil and transfer the contents into the bottom compartment of 18 test plates.

4. Hydrate the soil of 9 test plates with 35 ml distilled water (= control test plates) and the soil of the other 9 test plates with 35 ml boric acid solution (= toxicant test plates).

N.B. : The addition of 35 ml boric acid solution gives exactly a concentration of 250 mg boric acid/kg dry control soil.

5. Flatten the soil first in the control test plates and subsequently in the toxicant test plates with a spatula.

N.B. : An alternative procedure is to use the rectangular plastic strips and the flat bottom pestle (see Section 2.3.) for first flattening the dry soil, and subsequently hydrate it.

6. Label the 9 control test plates as follows :
   - LES control 1, LES control 2, LES control 3 for the test on *Lepidium sativum*
   - SIA control 1, SIA control 2, SIA control 3 for the test on *Sinapis alba*
   - SOS control 1, SOS control 2, SOS control 3 for the test on *Sorghum saccharatum*

7. Label the 9 toxicant test plates as follows :
   - LES tox 1, LES tox 2, LES tox 3 for the test on *Lepidium sativum*
   - SIA tox 1, SIA tox 2, SIA tox 3 for the test on *Sinapis alba*
   - SOS tox 1, SOS tox 2, SOS tox 3 for the test on *Sorghum saccharatum*

8. Put a black filter paper on top of the hydrated soil in each test plate.

9. Place 10 seeds in the 3 control plates and in the 3 toxicant plates for each of the 3 plant species.

10. Cover the test plates and put them vertically in the cardboard holders.

11. Incubate the test plates for 3 days at 25 °C (+/- 1 °C) in darkness.

12. Record the number of germinated seeds and make the length measurements of the roots in each test plate.

13. Score the data on the Results Sheet and calculate the percentage inhibition of the germination and the root length.

Data analysis and validity of the reference test

The 2 validity criteria for the Phytotoxkit assay, i.e. a mean % germination of at least 70% and a mean root length in the controls of at least 30 mm for the 3 test species (or 40 mm in case of measurement of the longest root) must also be fulfilled for the reference test.

From the data analysis of the International Interlaboratory Comparison of the Phytotoxkit, 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 have been calculated, and are given in the table hereunder for the 3 plant test species.

N.B. : The ringtest revealed that at a concentration of 250 mg boric acid/kg control soil there is no inhibition of the germination of the seeds.

The following 2 tables also show the "95% confidence limits" for the figures on the mean % inhibition of the root length. The latter ranges shall be considered as the "acceptance limits" for a Phytotoxkit reference test with 250 mg boric acid/kg control soil.

	Mean % inhibition of the mean root length	Acceptance limits (%)
Lepidium sativum	45	28 - 62
Sinapis alba	44	22 - 65
Sorghum saccharatum	30	9 - 52

	Mean % inhibition of the mean length of the longest root	Acceptance limits (%)
Lepidium sativum	42	27 – 58
Sinapis alba	44	28 – 60
Sorghum saccharatum	41	27 - 56

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LIST OF TOXKIT MICROBIOTESTS

Tests for freshwater and soils

PROTOXKIT F : 24h reproduction inhibition test based on the ciliate protozoan *Tetrahymena thermophila*. This assay is under consideration as an OECD Guideline.

ROTOXKIT F : 24h mortality test, based on the rotifer *Brachionus calyciflorus*. This assay adheres to ASTM Standard Guide E1440-91.

ROTOXKIT F short chronic : 48h reproduction inhibition test based on the rotifer *Brachionus calyciflorus*. This assay adheres to ISO norm 20666 and AFNOR norm T90-377.

THAMNOTOXKIT F : 24h mortality test, based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

DAPHTOXKIT F magna : 24h-48h mobility inhibition test, based on the cladoceran crustacean *Daphnia magna*. This assay adheres to ISO norm 6341 and OECD Guideline 202.

CERIODAPHTOXKIT F : 24h mortality test, based on the cladoceran crustacean *Ceriodaphnia dubia*. This assay is in current practice in the USA as an EPA Method.

OSTRACODTOXKIT F : 6 days chronic mortality and growth inhibition test with the ostracod crustacean *Heterocypris incongruens*. This assay adheres to ISO norm 14370.

RAPIDTOXKIT F : 30-60 min particle ingestion inhibition test based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

ALGALTOXKIT F : 72h growth inhibition test, based on the green alga *Selenastrum capricornutum* (presently named *Pseudokirchneriella subcapitata*). This assay adheres to ISO norm 8692 and OECD Guideline 201.

PHYTOTOXKIT : 3 days germination and root growth inhibition test with seeds of 3 higher plants.

PHYTOTESTKIT : A short germination and root/shoot growth inhibition microbiotest for determination of the direct effect of chemicals on higher plants.

SPIRODELA DUCKWEED TOXKIT : 72h growth inhibition test with the duckweed species *Spirodela polyrhiza*.

Tests for estuarine/marine environments

ROTOXKIT M : 24h mortality test based on the rotifer *Brachionus plicatilis*. This assay adheres to ASTM Standard Guide E1440-91.

ARTOXKIT M : 24h mortality test based on the anostracan crustacean *Artemia salina* (renamed *Artemia franciscana*). This assay adheres to ASTM Standard Guide E1440-91.

MARINE ALGALTOXKIT : 72h growth inhibition test based on the marine diatom *Phaeodactylum tricornutum*. This test adheres to ISO norm 10253.

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