P H Y T O T O X K I T

RAPID AND USER-FRIENDLY MICROBIOTEST
WITH HIGHER PLANTS

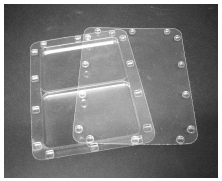
BENCH PROTOCOL

PRINCIPLE AND FEATURES

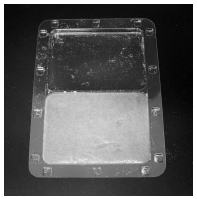
The Phytotoxkit microbiotest measures the decrease (or the absence) of germination and of the growth of the young roots after a few days of exposure of seeds of selected higher plants to toxicants or to contaminated soils, in comparison to the controls in a reference soil.
Besides toxicity assessment 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.

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

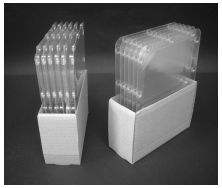
The Figures hereunder illustrates the test concept of the Phytotoxkit.



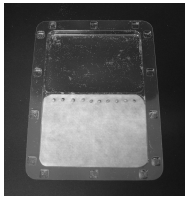
test plate with cover



filling of bottom compartment with soil, and hydration



vertical incubation of the test plates in the holder



placing of filter paper and seeds, and closing of the test plates

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 both “plant-dependent”.
At the end of the incubation period a “digital” picture is taken of the test plates with the germinated plants, for storage in a computer file. The analyses and measurements by image analysis can be made either immediately or postponed to any appropriate timing.
The application of the Phytotoxkit microbiotest is very flexible and allows in principle to use any type of plant seed and test soil or substrate, in comparison to a control soil.

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 for a reference test (quality control test) with boric acid

Three plant species have been selected for the standard Phytotoxkit microbiotest on the basis of the rapid germination of the seeds and growth of the roots, which allows to complete the assay after only 3 days of incubation: the monocotyl Sorgo (*Sorghum saccharatum*) and the dicotyls garden cress (*Lepidium sativum*) and mustard (*Sinapis alba*).

The standard Phytotoxkit measures two kinds of effects : the decrease of seed germination and the decrease of root growth* in comparison to germination and root growth in a control soil.

* *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”.*

N.B. Various other effects 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, in parallel to 3 replicates on a control soil (a modified OECD artificial soil composed of sand, kaolin and peat).

A “Phytotoxkit accessories package” can be obtained separately for easy test preparation.

TEST PROCEDURE

1. Quick method for determination of the water holding capacity (WHC) of the test soils

Phytotoxkit assays are carried out at “water saturation” of the soils at the start of the tests

A. Control soil

The WHC of the control soil has been determined experimentally. 35 ml water must be added to the 90 cm^3 soil in the test plates to obtain 100% water saturation.

B. Test soil

Air-dry about 1 dm^3 of test soil (by spreading the soil in a thin layer on a flat surface) until is 'pulverises' easily on pressing it between thumb and finger. Sieve the air-dried soil through a sieve with a 2 mm mesh to eliminate all coarse material. Fill a 100 ml beaker to the 90 ml mark with soil and slowly add 50 ml distilled (or deionised) water. Mix the water thoroughly with the soil till all the soil is wet. Wait for the soil/water mixture to reach equilibrium leading to a water-saturated soil phase with a water layer on top.

Take the microsieve cylinder and bring it down vertically in the beaker with water-saturated soil. With the aid of the wide mouth micropipette suck up the water from the inside of the microsieve cylinder and transfer it into the graduated cylinder. Repeat this operation, pushing the microsieve cylinder further down into the soil until no water penetrates anymore into the microsieve cylinder. 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 ml – S ml). Discard the hydrated 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

A. Control soil

Take one bag with (90 cm^3) reference sand and pour the contents in the lower compartment of a test plate. Fill a 50 ml syringe with distilled water to the 35 ml mark. Hold the syringe vertically above the soil in the test plate and slowly drop out the water over the whole surface of the soil.. With the aid of a spatula spread the wet soil evenly over the total surface of the soil and flatten the surface to obtain a soil layer of uniform depth. Repeat the former operations for the 8 other control test plates.

B. Test soil

Fill the 100 ml beaker to the 90 ml mark with the sieved test soil and transfer the soil to the bottom compartment of a test plate. Hydrate the test soil the same way as indicated for the control soil, by addition of a volume of distilled water = Vsat. Use the spatula to spread the soil evenly over the total surface of the bottom compartment of the test plate. Repeat the former operations for the 8 other plates with test soil.

N.B. : A time saving alternative for flattening of the soil, and which also prevents spilling of soil, is to use the rectangular plastic strips and the small flat bottom pestle which are included in the Phytotoxkit accessories package.

3. Placing of the filter paper and the seeds, and closing of the test plates

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.

Place 10 seeds (of the same test plant) on top of the filter paper in one row near the middle ridge of the test plate and at equal distance of each other. The test plates are provided with marks to facilitate the correct positioning of the seeds. Carefully place the cover on the bottom part of the test plate 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.

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

4. Incubation of the test plates

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

5. Image recording at the end of the exposure period

The pictures of the test plates at the end of the exposure period can be taken either with a digital camera, a webcam camera or a flatbed paper scanner. Any type of image analysis programme can be used for the subsequent analysis, provided it allows for length measurements. A convenient and practical programme is "ImageJ" which can be downloaded from the Internet or obtained free of charge from MicroBioTests Inc. The recorded pictures shall be stored in a file with a JPEG extension in the selected Directory.

N.B. If condensation occurs on the inside of the lid of the test plate (interfering with the visibility of the roots), the lid shall be (carefully) separated from the bottom of the test plate to wipe off the condensation.

6. Analysis and measurements of the germinated seeds in the stored files

A. Counting of the number of germinated seeds

Open the files with the recorded images and count the number of germinated seeds in each test plate. Note down the figures in the Results Sheet in Section "A. Seed Germination".

B. Measurement of the root lengths of the germinated seeds

Length measurement of the roots shall be made following the specifics of the selected image analysis programme. Write down the mean root length for the germinated seeds in each test plate in the section "B. Root Growth" of the Result Sheet.

N.B. : A "substantial time saving" alternative is to measure only the length of "the longest root" in each test plate. Extensive research has shown that calculations made on the basis of only the length of the longest root in each test plate gives basically the same inhibition effect percentages as with calculations based on the length measurement of all the roots.

7. Calculation of the percentage inhibition of seed germination and root growth

With the data noted down in the Results Sheet, calculate the mean number of germinated seeds and the mean root length for each test plant, for the 3 test plates with control soil and for test soil.

Subsequently calculate the percentage inhibition of seed germination and root growth inhibition for each plant with the formula : (A – B)/A * 100

with A = mean seed germination or root length in the control soil
and B = mean seed germination or root length in the test soil

N.B. : A programme is available which automatically calculates the percentage inhibition of seed germination and root lengths. This programme can be obtained free of charge from MicroBioTests Inc.

8. Validity criteria

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 of measurement of only the longest root, the minimum mean length in the control test plates must be at least 40 mm for each of the 3 plants test species.

9. Reference test

A quality control test can be carried out with one concentration of the reference chemical boric acid (250mg/kg reference soil). The procedure for the reference test is described in detail in the Phytotoxkit Standard Operational Procedure.