P H Y T O T E S T K I T

For determination of the direct effects of chemicals
on seed germination and early growth of plants

LIMIT **TEST WITH 3 PLANT SPECIES**

PRINCIPLE AND FEATURES

The **Phytotestkit** microbiotest is a variant of the **Phytotoxkit** assay which measures the decrease (or the absence) of germination and early growth of plants in contaminated soils, in comparison to the germination and growth in a reference soil.
Both the Phytotoxkit and the Phytotestkit microbiotests allow for direct length measurements of roots and shoots in special transparent test containers, with the aid of image analysis, which eliminates the time consuming manipulations inherent to conventional phytotoxicity assays in pots.
In the **Phytotestkit test** the lower compartment of the test plate is not filled anymore with soil, but with a foam pad and a thick filter paper which is subsequently spiked with **one** selected concentration of a chemical compound. This alternative test procedure allows to determine the direct intrinsic effect of a chemical compound (at the selected concentration) on the plant.

The Phytotestkit is available in two versions :

1. ***The Phytotestkit complete test*** *for performance of a "complete test" with 5 test concentrations of a chemical compound, on one plant species, and which allows to calculate the EC50.*
2. ***The Phytotestkit limit test*** *for performance of the assay on 3 test species, at one concentration of the chemical compound.*

The Phytotestkit limit test detailed hereunder contains tubes with 3 types of plants seeds which were selected for their rapid germination and growth of the roots and shoots, which allows to complete the assay after only 3 days of incubation: the monocotyl Sorgho (*Sorghum saccharatum*) and the dicotyls garden cress (*Lepidium sativum*) and mustard (*Sinapis alba*).

Seeds of the 3 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 spiked thick white filter paper. After closing the test plates with their transparent cover, the test plates are placed vertically in a holder and incubated at 25 °C (+/- 1 °C) for 3 days.
At the end of the incubation period a "digital" picture is taken of the test plates in which the germinated plants can clearly be seen underneath the transparent cover. The pictures are stored in a computer file for subsequent analyses and length measurements of the roots and the shoots.

The Phytotoxkit limit test allows to perform a bioassay with each of the 3 plants mentioned above, and with 3 replicates, at one (selected) concentration of a chemical, in comparison to a control.

The Phytotestkit limit test can also be applied with other types of seeds and can also be used to study the dynamics of early plant growth and for analysis of (positive or negative) effects of soil fertilisers, agricultural chemicals or biocides. Pictures of the growing plants in the transparent test plates can indeed be taken "at any moment of time" during the entire incubation period.

ASSETS OF THE PHYTOTOXKIT

The Phytotestkit has multiple advantages over conventional bioassays with higher plants :

- the technology allows to determine the "intrinsic" (direct) effects of chemicals on germination and early growth of plants without interference of a soil substrate
- the test plates are small and require little bench space and incubation space
- the transparent test plates allow for direct observation of the germinated seeds and the growing plants without any manipulation
- the pictures of the test plates with the germinated seeds are stored in computer files, which allows to postpone the measurements
- the length measurements of the roots and shoots is rapid and automatic by the use of image analysis techniques
- this microbiotest also allows to study the early growth dynamics of the plants

The only equipment needed for performance of Phytotestkit tests is an incubator (or a temperature controlled room) and a digital camera (or a webcam camera or flatbed paper scanner) for the shooting of the pictures of the test plates with the germinated seeds and for the subsequent storage in a computer file. The length measurements of the roots and the shoots are made with an Image analysis programme.

TEST PROCEDURE

The procedure described hereunder outlines the handlings on one test plate (see Figure 1), with spiking of the white filter paper with one concentration of a chemical compound (and with deionized or distilled water for the control test plate).

1. Filling of the test plate with foam pad, Parafilm sheet and white filter paper, and spiking with test compound

Put one foam pad in the bottom compartment of a test plate, place a Parafilm sheet on top and put one white filter paper on top of the Parafilm sheet (*see Figure 2*).
Fill a syringe with 20 ml of the selected chemical solution and slowly spread it over the entire surface of the white filter paper to hydrate the latter completely (*see Figure 3*).

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

Put one thin black filter paper on top of the hydrated white filter paper and wait until the black filter is completely wet (*see Figure 4*)

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

Place 10 seeds of the selected test plant on top of the black 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 (*see Figure 5*).

Carefully place the cover on the bottom part of the test plate (*see Figure 6*) and click the bulges of the side of the cover into the corresponding cavities of the bottom part to close the test plate tightly

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

Write the specifics of the test plate (name of the plant species and number of the replicate) on one of the small labels and stick this label laterally on the bottom compartment of the test plate, in between two rectangular cavities.
Turn the closed test plate upside down for a few hours to facilitate the hydration of the seeds.

3. Incubation of the test plates

Put the test plates in a vertical position in the cardboard holders, with 6 test plates per holder.
Put the cardboard holders with the test plates in the incubator and incubate at 25 °C (+/- 1 °C) for 72h.

N.B. Light does not seem to play a significant role for the germination of the seeds nor the growth of the roots and shoots during the incubation time of only a few days. It is therefore advised not to provide illumination during the test period unless one wants to specifically study the influence of light, especially with longer exposure times.

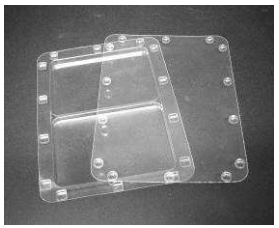

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

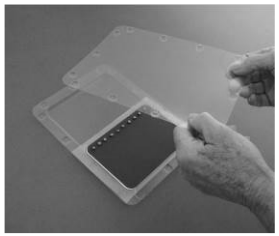

Figure 6

4. Image recording at the end of the exposure period

As indicated above, 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 at no charge from the company MicroBioTests.

A. Image recording with a webcam camera
The webcam camera shall be fixed to a stand and the test plates placed horizontally on a flat surface under the camera. The procedure specific to the webcam camera shall be followed for the image recording and the pictures stored in a file with a JPEG extension in the selected Directory.
B. Image recording with a digital camera
The digital camera can be mounted on a vertical stand, or a stand with a telescopic arm, and depending of 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). The shot pictures shall be transferred to the computer and stored as JPEG files.
C. Image recording with a flatbed paper scanner
Put the test plate "surface down" on the paper scanner. Put the lid of the paper scanner on the test plate and record the image with the appropriate programme. Give the file a name (with a JPEG extension) and store it 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 lids shall be (carefully) separated from the bottom of the test plate to wipe off the condensation prior to shooting the picture.

5. 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 and calculate the mean values for the 3 replicates for each of the 3 plant species.
B. Measurement of the root and shoot lengths
Length measurement of the roots and shoots shall be made following the specifics of the selected image analysis programme. Calculate and write down the mean root and shoot length for the germinated seeds in each test plate for the 3 plant species in the Result sheet.

6. Calculation of the percentage effect of the spiked chemical on seed germination and root and shoot growth

With the data of the Results Sheets, calculate the percentage effect of the chemical compound (at the test concentration used) on seed germination and root and shoot growth for each of the 3 plant species with the formula :

$$\frac{A-B}{A} \text{ X } 100$$

with A = average number of germinated seeds and average root and shoot length in the control and B = average number of germinated seeds and average root and shoot length of the chemical (at the selected test concentration).