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

BENCH PROTOCOL

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 assay 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 the chemical compound under analysis. This alternative test procedure allows to determine the intrinsic effect of chemical compounds on plants, without incorporation of the chemical(s) in a soil substrate.

Seeds of the selected test plant(s) are positioned at equal distance near the middle ridge of the test plate, on a black filter paper placed on top of the spiked filter paper. After closing the test plates with their transparent cover, the test plates are placed vertically in a holder and incubated at the selected temperature and for the selected incubation period.

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 and subsequent analyses and length measurements of the roots and the shoots.

The Phytotestkit test procedure is very flexible and allows to apply this test with any type of seed. In addition this microbiotest can also be used to study the dynamics of early plant growth in function of the concentration of the spiked chemical compound(s). 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 Phytotoxkit 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, which allows to perform multiple concurrent tests
- 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 is rapid and automatic by the use of image analysis techniques
- this microbiotest also allows to study the early growth dynamics of the plants

Like the Phytotoxkit the Phytotestkit contains tubes with 3 types of plants seeds which have been selected for their rapid germination and growth of the roots and shoots and allow 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*).

The Phytotestkit microbiotest measures two kinds of effects : a) the effect of the chemical compound on seed germination and b) the effects on early growth of the plan. Both effects are evaluated in comparison to the not spiked control

A Phytotoxkit contains 18 test plates, each provided with a foam pad, a white filter paper and a black filter paper and allows to set up a variety of experiments.

Conventional test procedure

This bioassay consists of a dilution series of 5 concentrations of a chemical (or mixture of chemicals) + one control, to be spiked on the white filter paper. The assay is carried out with one type of seed and with 3 replicates for each test concentration + the control. The 3 Results sheets included in the Phytotestkit have been made up specifically for this type of test procedure, for calculation of the percentage effect of the spiked chemical at each test concentration for the criteria "germination", "root length" and "shoot length". If desired, EC50's can be calculated.

Alternative test procedures

The conventional test procedure can also be applied with 3 types of seeds instead of one (e.g. the 3 seeds included in the kit), in which case there will only be one replicate per seed for each test concentration.

A multitude of other testing procedures can, however, also be applied, such as e.g. : use of a smaller or a larger number of concentrations of the spiked chemical, testing of the effect of different incubation temperatures on the impact of the chemical compound, or combination experiments with e.g. testing of the effect of a chemical at different temperatures

The only equipment needed for performance of the Phytotestkit microbiotest 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, for subsequent storage in a computer file. The length measurements of the roots and the shoots can be made with any image analysis programme.

TEST PROCEDURE

The procedure outlines the handlings on one test plate (see Figure 1), with spiking of the white filter paper with one concentration of a chemical compound.

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

Put one foam pad in the bottom compartment of a test plate, and put one white filter paper on top (*see Figure 2*)

Fill a syringe with 20 ml of the chemical solution under analysis and slowly spread it over the entire surface of the white filter paper to hydrate the filter paper completely (*see Figure 3*).

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

Put one 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 the paper 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*).

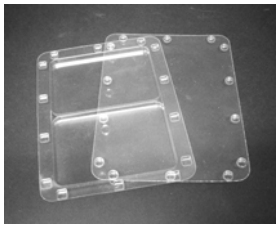

Figure 1

Figure 2

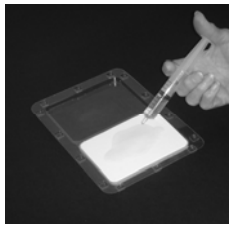

Figure 3

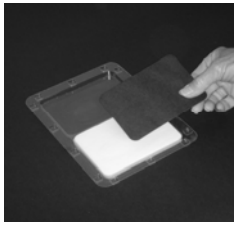

Figure 4

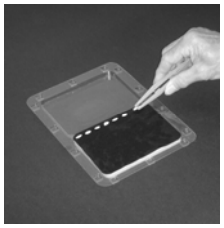

Figure 5

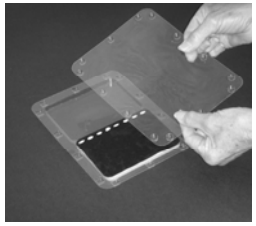

Figure 6

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 (type of seed, test concentration, 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 for the selected exposure time and at the selected temperature.

N.B.1. In case of use of the seeds provided in the Phytotestkit the recommended exposure time is 3 days and the incubation temperature 25°C.

N.B.2. 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.

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 "Image Tools" which can be downloaded directly from the Internet or obtained at no charge.

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

Proceed as for shooting and storing the image of the test plates with a webcam camera. The digital camera can also be mounted on a table top tripod stand for taking the pictures of the test plates, placed in this case vertically at the appropriate distance of the camera.

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 "Seed Germination" Results sheet.

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. Write down the mean root length for the germinated seeds in each test plate in the "Length of roots" and "Length of shoots" Result sheets.

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

As indicated above, the Results sheets included in the Phytotestkit are specific for a bioassay with 5 concentrations (+ one control) of a chemical compound, performed with one type of seed, and in 3 replicates per test concentration.

With the data of the Results Sheet, calculate the mean number of germinated seeds and the mean root and shoot length for the 3 replicates in the control and in each test concentration.

Subsequently calculate the percentage effect of the chemical compound on seed germination and root and shoot growth 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 in the 5 test concentrations