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A Practical and Low Cost Microbiotest to Assess the Phytotoxic Potential of Growing Media and Soil

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Abstract

For routine toxicity testing of composts and growing media, two different types of assays are usually applied. One approach is the use of growing trials in pots with various mixtures of the material. In extract-based tests, seeds are germinated in petri dishes and exposed to an exudate from the material to be tested. In this study, one of these methods, the Phytotoxkit microbiotest, was compared to the standard phytotoxicity test with *Lactuca sativa* as used by the RHP foundation. The Phytotoxkit test was performed in transparent test containers which allow for direct observation and length measurements of the seedlings by means of image analysis. Two dicotyles (*Lepidium sativum* and *Sinapis alba*) and one monocotyle (*Sorghum saccharatum*) were used. When measuring a presumably phytotoxic bark, in comparison with a reference bark, the Phytotoxkit showed a seedling length inhibition of 60–80% for the dicotyles and less than 20% for the monocotyle after three days. The RHP growing trial showed 30% weight inhibition of the lettuce after two weeks of plant growth when comparing the same barks in mixtures. This preliminary comparison showed that the Phytotoxkit microbiotest is a quick and practical bioassay with a high resolution which has a potential to become an international standard.

INTRODUCTION

Testing growing media and soil improvers on the presence of harmful components is important for quality control and risk reduction in the process of producing potting soils and container media. In liability cases, prior phytotoxicity testing of all the components used in the production of container media is often regarded as a prerequisite to prove careful production.

Phytotoxicity tests may involve growing trials, derived from methods used for potting soil and peat (Verhagen, 2000; Morel and Guillemain, 2004). In such tests, a material is mixed in various dilutions with e.g., peat and used in a randomized design container culture trial. Generally two or three plant species from different families are tested, because plants differ widely in their response to different toxins (Wang, 1991).

These growing trials are regarded as reliable, but have some disadvantages. They are relatively time consuming, because several weeks to months may be involved. Also, the structure of the growing medium used may influence the plant's response, and poor structure may be mistaken for toxicity. The mixing of the material with substances as peat results in dilution of the toxin in the root zone. Furthermore, the experiments may start with a plantlet grown in another medium than the mixture tested, which creates chance on interaction. The comparison of results is sometimes difficult, because light levels, temperature and humidity may vary from test to test.

Another approach to test for phytotoxicity uses seedling shoot growth in a petri dish as a parameter for toxicity (Fig. 1). These tests are derived from methods for soils, composts and growing media (US EPA, 1996; Baudo et al., 1999; ISTA, 2005). In these tests, some 10 seeds in a few replicates are germinated in petri dishes on wetted filter paper, and exposed to an exudate subtracted from the medium to be tested. After a few days incubation period, the seedlings are stretched out for manual length measurement.

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These tests are highly sensitive, because of the plants seedling stage. They are generally regarded as reliable and fairly rapid. Disadvantages are the time required for measurements, the water supply and precision. As the seedlings will try to grow vertically in the petri dish, they will become twisted (Fig. 2). The subsequent stretching of the shoot for measurement is time consuming. Furthermore, care must be taken that the water uptake during the germination process is not limited by the maximum amount of water present in the filter paper. The precision of the petri dish assay is inter- and intra-laboratory dependent, as shown by several intercalibration exercises (Beltrami et al., 2002).

It is clear that for assessing the phytotoxic potential of growing media and soil, each of the two approaches has specific advantages and weaknesses. Therefore, a new bioassay, the Phytotoxkit (Phytotoxkit, 2004) was tested. This test is performed in flat transparent containers which only allow seedling growth in one direction (Fig. 3). The seeds are on top of a filter paper which is placed on the wetted substrate. The test containers are incubated in vertical position to allow for normal seedling development. After the incubation period, seedling growth can be recorded with a digital camera, which allows for subsequent measurement by image analysis. In order to evaluate the potential of the Phytotoxkit, we compared this bioassay with a standard growing test (Verhagen, 2000).

MATERIAL AND METHODS

Standard RHP Phytotoxicity Test with Lettuce

As a reference 15 L of a mixture was made. The on e component was 75% v/v of a white peat used as standard in the laboratory involved. The other component was 25% v/v of a known non toxic reference bark from the laboratory store. Another mixture of 15 L was made of the standard white peat, 75% v/v, with 25 v% of a presumably phytotoxic bark material, PPBM. The mixtures were prepared under standardised compaction, and mixed with powdered calcium carbonate to reach a pH between 5.0 and 5.5. Fertilizer was added in the form of 0.75 g of PG-mix per litre. Water (EC < 0.1 $dS.m^{-1}$) was added to a content of approximately 50% v/v (showing water release on pressing the sample by hand). The mixtures were stored for at least 24 hours at 20°C in the dark, in closed plastic bags. The test was carried out with lettuce (*Lactuca sativa*).

1. Test Procedure. Per mixture, four replicate sample trays were filled (surface area 0.075 m^2 each). Per tray, 3.0 L of the mixture and 500 ml of water were added. Each sample tray was slightly compressed as prescribed and 3 rows of ten holes, 5 mm depth, were made in the flattened substrate. Each tray was then sowed with 30 lettuce seeds. The trays were stored for 18–24 hours at 12°C. After that period the trays were placed in a plastic tunnel in a greenhouse at 22/20°C (day/night) and RH > 80%. Up to 14 DAS (Days After Sowing) the trays were remoistened every two days. At 7 DAS, germination was scored positive if two lobes were visible; at 14 DAS the above ground fresh weight and the number of plants per tray were measured. Growth depression was reported as ((APWref – APWtest)/APWref) * 100), APW being the average plant weight which is total plant weight divided by number of plants. In formula APWtest = TPWtest / Ntest and APWref = TPWref / Nref, TPW being the total weigh of all plants and N the number of plants weighed.

Phytotoxkit Microbiotest

The Phytotoxkit tests (MicroBioTests Inc.) were performed according to the Standard Operational Procedure of this assay (Phytotoxkit, 2004) and with the materials included in the commercial Phytotoxkit. The materials to be tested were a presumably phytotoxic bark material and a known non toxic reference bark from the laboratory store. Both materials were used to fill a 1200 ml beaker. Dry weight was established before slow submergence in demineralised water. After 24 hours at 20°C, the material was allowed to drain to equilibrium on a grid and the saturated weight was measured. All

assays were carried out with seeds of three different plant species, the monocotyle *Sorghum saccharatum* (sorghum), and the dicotyles *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard).

1. Test Procedure. 90 ml of the presumably phytotoxic bark material was transferred to the bottom compartment of a 21 cm x 16 cm test plate, and hydrated with the volume of distilled water calculated as necessary to reach saturation of the substrate. A reference test plate was filled with the reference bark (Fig. 4). The wet bark in the test plates was then flattened with a spatula and covered with a 0.5 mm thick filter paper. Ten seeds of one species were subsequently placed on the filter paper in a single row, close to the dividing ridge. All assays were performed in 3 replicates for each of the three plant species used. Then the test plates were closed with the transparent lid. The test plates were placed vertically in the cardboard holder and incubated for 3 days at 25°C, in darkness. At the end of the incubation period a picture of each test plate was taken with a digital camera. Root and shoot length measurements were made subsequently with the aid of an image analysis programme. A second series of pictures of the test containers was taken after 7 days incubation to assess seedling growth after prolonged exposure.

RESULTS AND DISCUSSION

For the RHP cultivation test, the germination counts at 7 and 14 DAS and fresh weight data per tray and per germinated plant at 14 DAS are shown in Table 1. Photos were taken at 14 DAS. At 7 DAS the germination in the PPBM mixture was 82% but 97% in the reference mixture. At 14 DAS the number of germinated plants per tray had become equal. The total weight of the plants grown in the PPBM mixture was 15.3 g, significantly lower than the 22.2 g in the reference mixture. Expressed as the above ground weight reduction, the PPBM mixture reduced weigh growth with 30% g/g as compared to the reference mixture.

For the Phytotoxkit bio-assay digital images were taken at 3 DAS and at 7 DAS. The digital images were used for the germination count and root and shoot length measurement (3 DAS) and root and shoot length measurement (7 DAS, Table 2).

For the Phytotoxkit, 3 DAS, the response of mustard was the strongest with 81% shoot length inhibition and 73% root length inhibition in the PPBM as compared to the reference bark (Fig. 5). Garden cress showed 61% shoot length inhibition and 65% root length inhibition in the PPBM as compared to the reference bark. Sorghum showed the weakest response with 17% shoot length inhibition and 7% root length inhibition in the PPBM as compared to the reference bark.

In most cases the response of the shoots is stronger then that of the roots. This may be caused by the food reserve in the seed that is used primarily for the growth of the root. The response after 7 days is similar but less distinctive for mustard and cress and clearer for sorghum. This might reflect the longer growth time to maximum height for sorghum.

The overall accuracy (l.s.d. at p=0.05) of the microbiotest is less if compared to the reference test. But the magnitude of growth reduction is higher in the microbiotest. The number of replicates in the standard Phytotoxkit assay is 3 while the RHP test has 4 replicates.

For a comparison of methods with such principal differences as different rooting media, different mixtures of the suspected bark, different plant species and a different number of replicates, a further validation to establish the correlation between the two methods seems desirable. Such a trial could be made by testing 3–5 concentrations of a toxic substance in both methods with the same number of replicates.

CONCLUSIONS

The Phytotoxkit is a competitive extract based method with the possibility to use the percentage of germination and the seedling growth. The seeds germinate under a stable water supply and the measurements are easy and suitable for image analysis. The growth inhibition of the dicotyledonous plants in the Phytotoxkit was about twice that of the lettuce in the standard growing trial. The number of replicates should preferably be at

least 4 instead of 3. Probably the detection level of the standard growing test can be surpassed by the Phytotoxkit, because it shows a higher growth reduction. The Phytotoxkit method has potential to become an international standard.

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Tables

Table 1. Mean count a
above ground part
14 DAS in a PPBM

Mixture	DAS 7 germinatio (%)
PPBM	81.8
Reference	96.7

[1] DAS; days after sowing
[2] PPBM; probably phytoto
[3] Calculated as ((FWref –
[4] Least Significant Differe

Table 2. Mean lengt
compared to refer
species, n=3.

	Time
Shoot length	DAS 3
	DAS 7
Root length	DAS 3
	DAS 7

[1] DAS; days after sowing
[2] PPBM; probably phytot
[3] Calculated as ((mm-ref
a,b Least Significant Diff
bark (not shown) being

Tables

Table 1. Mean count and percentage of germinated plants, mean fresh weight (FW) of the above ground parts and mean percentage weight reduction at 7 DAS[1] respectively 14 DAS in a PPBM[2] mixture and a reference mixture for lettuce in the RHP test, n=4.

Mixture	DAS 7 germination (%)	DAS 14 FW/tray (g)	DAS 14 Plants/tray (nr)	DAS 14 FW / plant (g)	DAS 14 growth reduction[3] (% g/g)
PPBM	81.8	15.3	89.3	0.17	30.3 b[4]
Reference	96.7	22.2	90.0	0.25	100.0 a[4]

[1] DAS; days after sowing
[2] PPBM; probably phytotoxic bark material
[3] Calculated as ((FWref – FWppbm)/FWref) * 100)
[4] Least Significant Difference of means, at the p=0.05 level, is 10%

Table 2. Mean length of the shoots and the roots in mm and in% length inhibition compared to reference bark at 3 and 7 DAS[1)] in PPBM[2)] for the 3 Phytotoxkit test species, n=3.

	Time	(mm)	Cress (% g/g[3])	(mm	Mustard (% g/g[3])	(mm)	Sorghum % g/g[3])
Shoot length	DAS 3	10.8	61.0 b	2.7	80.8 b	8.6	17.2 b
	DAS 7	27.7	41.1 b	26.7	65.4 b	64.1	39.9 b
Root length	DAS 3	17.1	64.7 b	13.1	72.6 b	33.3	6.7 a
	DAS 7	*	*	29.0	61.6 b	66.0	19.4 b

[1] DAS; days after sowing stands for days after incubation
[2] PPBM; probably phytotoxic bark material
[3] Calculated as ((mm-ref – mm-ppbm)/mm-ref) * 100)
a,b Least Significant Difference of means, at the p=0.05 level, is 12.2% between substrates, the reference bark (not shown) being 100% a at all times

Figures

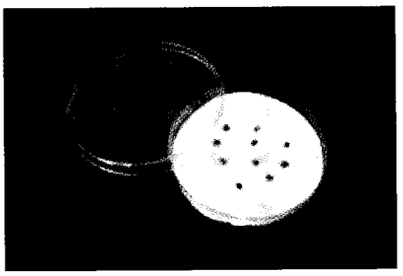


Fig. 1. Petri dish with filter paper and seeds.

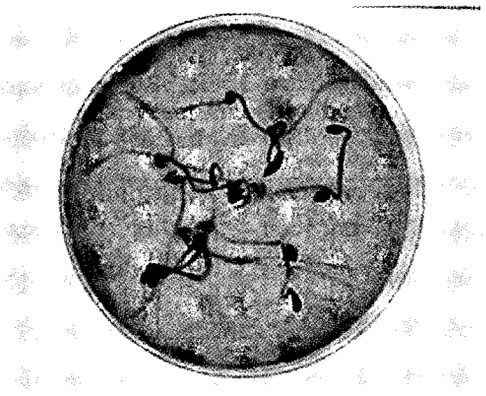


Fig. 2. Petri dish with germinated seeds after a few days of incubation.

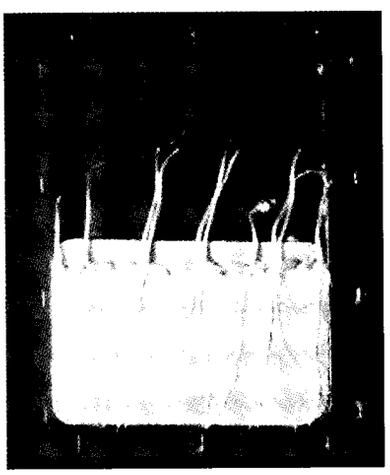
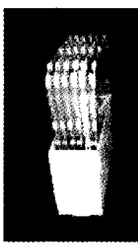


Fig. 3. Transparent test plate with germinated cucumber seeds after a few days of incubation.

Test plate



Vertical incul
testplate

Fig. 4. Phytotoxk

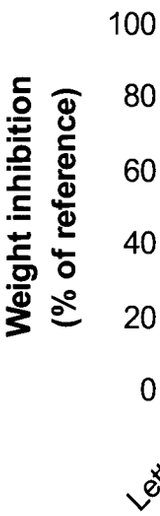


Fig. 5. Bar graph
RHP grow
DAS 3 on
mixture (l
axis).

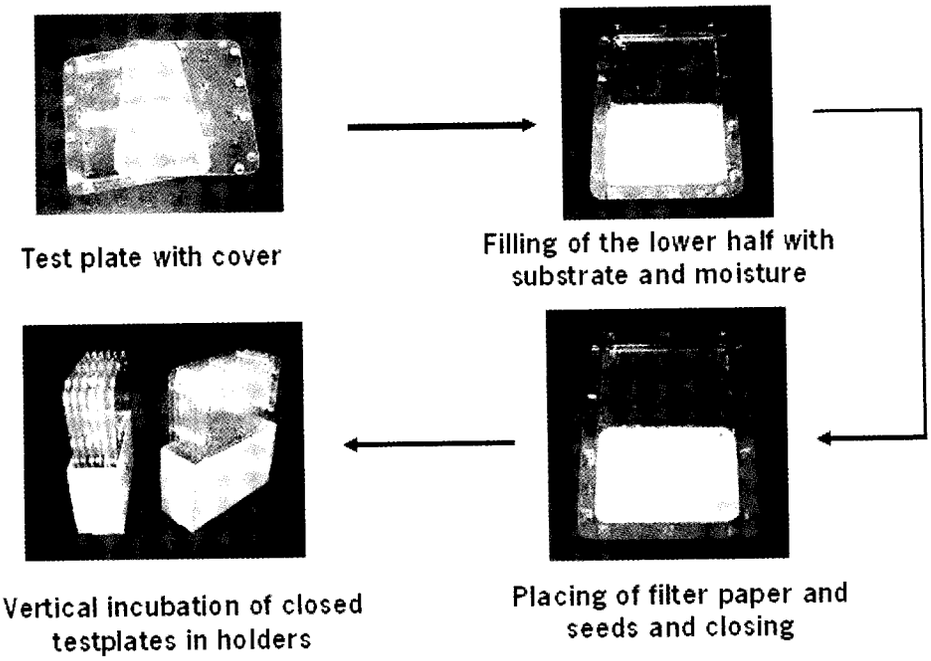


Fig. 4. Phytotoxkit test procedure.

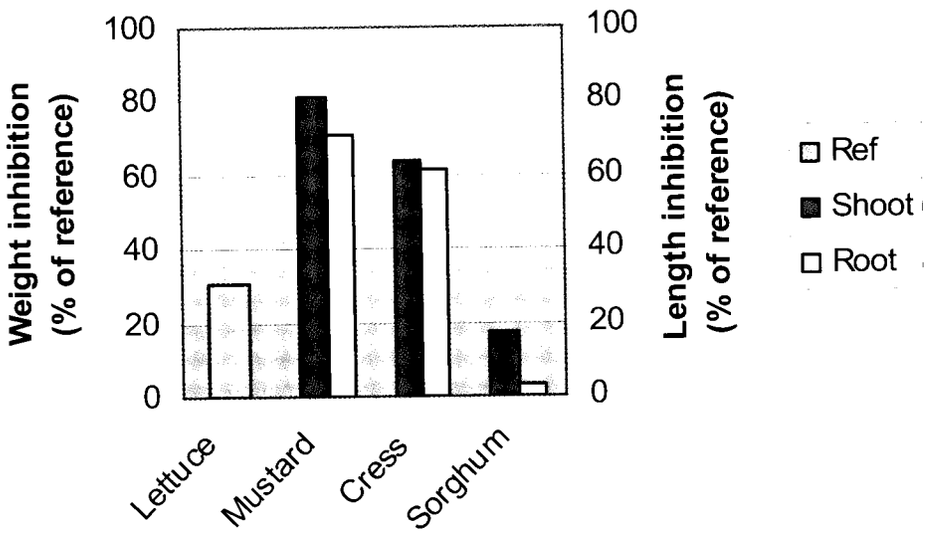


Fig. 5. Bar graph comparing two phytotoxicity tests. Left hand bar represents the standard RHP growing test. Right hand side bars show the results of the Phytotoxkit test, DAS 3 only. Bar length shows either weight inhibition in percentage of reference mixture (left y-axis) or length inhibition in percentage of reference bark (right y-axis).