

# Isolation of *Cylindrocladium* spp. in roots and soils from banana cropping systems

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## Isolation of *Cylindrocladium* spp. in roots and soils from banana cropping systems.

**Abstract — Introduction.** This protocol aims at detecting *Cylindrocladium* spp. in root and soil samples from banana fields and at quantifying the inoculum. The principle of the two methods applied [Root Slice Plating Method (RSPM) and Soil Isolation Method (SIM)], the key advantages of these methods, starting plant and soil materials, the time required and the expected results are presented. **Materials and methods.** Necessary laboratory materials, and details of the six steps achieved for the use of the two methods are described. **Results.** When using the RSPM, typical *Cylindrocladium* colonies are isolated in petri dishes. When using the SIM, typical *Cylindrocladium* colonies originate from microsclerotia embedded or not in banana root tissue. The inoculum density can be calculated.

France (Guadeloupe) / *Musa* sp. / disease control / methods / laboratory equipment / *Cylindrocladium* spp. / inoculation / roots / soil

## Isolation de *Cylindrocladium* spp. à partir de racines de bananier et de sols de bananeraies.

**Résumé — Introduction.** Ce protocole vise à détecter *Cylindrocladium* spp. dans des échantillons de racines et de sol de bananeraie et à en mesurer l'inoculum. Le principe des deux méthodes appliquées [méthode d'étalement de lamelles de racines (RSPM) et méthode d'isolement de sol (SIM)], les principaux avantages de ces méthodes, le matériel végétal et les échantillons de sols utilisés, le temps nécessaire et les résultats attendus sont présentés. **Matériel et méthodes.** Le matériel de laboratoire nécessaire et le détail des six étapes réalisées pour appliquer les deux méthodes sont décrits. **Résultats.** En appliquant la méthode RSPM, des colonies types de *Cylindrocladium* sont isolées en boîtes de Pétri. Par utilisation de la méthode SIM, les colonies types de *Cylindrocladium* sont obtenues de microsclérotés incluses ou non dans le tissu des racines de bananier. La densité d'inoculum peut être calculée.

France (Guadeloupe) / *Musa* sp. / contrôle de maladies / méthode / matériel de laboratoire / *Cylindrocladium* spp. / inoculation / racine / sol

## 1. Introduction

### Application

This protocol aims at detecting *Cylindrocladium* spp. in root and soil samples from banana fields and at quantifying the inoculum.

### Principle

Two methods are applied:

(a) Root Slice Plating Method (RSPM): roots are thoroughly washed under a tap to

remove soil. Fragments bearing necrotic lesions are then selected and externally disinfected. Root slices are aseptically cut at the junction of healthy and necrosed tissues and plated onto M1–100 CHL agar isolation medium. When present, typical *Cylindrocladium* colonies readily appear after 3–4 days and can be counted.

(b) Soil Isolation Method (SIM): soil samples from banana fields are processed using a combination of blending, washing and wet-sieving techniques. Resulting residues are then quickly disinfected before incor-

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poration into an agar selective isolation medium that favours growth of *Cylindrocladium* colonies while restraining that of other soil fungi. With respect to the dry weight of a similar soil sample, occurrence and quantification in soil of *Cylindrocladium* fungi can be evaluated and expressed as *Cylindrocladium* inoculum densities in soils (number of colony-forming units per g of dry soil).

### Key advantages

Each of the two methods used presents advantages:

(a) The RSPM is easy to process; it does not require any special or expensive equipment; it is suitable for routine monitoring or field surveys to detect the presence of *Cylindrocladium* in banana plots, as many root samples can be processed simultaneously.

(b) The SIM, derived from those of Phipps *et al.* [1] and Risède [2], allows spatiotemporal field monitoring of the distribution and persistence of *Cylindrocladium* fungi in soils without any special equipment such as elutriators; in comparison with *Cylindrocladium* baiting isolation methods [3], it enables quantification of *Cylindrocladium* inoculum in soils.

### Starting material

The methods require root and soil samples from banana cropping systems. Root samples are preferably collected from ten or more banana plants and then mixed. Soil subsamples are preferably harvested in the rows from ten or more locations within the plot before being pooled and mixed to obtain a representative soil sample for the plot.

### Time estimation

For the RSPM, the time is variable, depending on the amount of roots to be processed. For a well-trained operator, fifty root isolations will take about 2–2.5 h once the isolation medium dishes are available. For the SIM: four soil samples can be processed in about 3–3.5 h. Five to eight days are then necessary to allow the development of

*Cylindrocladium* colonies in the agar isolation medium dishes.

### Expected results

Isolation petri dishes are examined to detect the presence of *Cylindrocladium* fungi in root or soil samples. The RSPM also enables estimation of the root isolation frequency of *Cylindrocladium* fungi in the banana plot as well as the extent of these fungi relative to the total mycoflora associated with root lesions. The SIM also enables estimation of the *Cylindrocladium* inoculum density in the soil of the studied plot. In all cases, when *Cylindrocladium* strains are detected, they can be kept for further diagnostic analyses such as species determination or pathogenicity studies.

## 2. Materials and methods

### Laboratory materials

Both methods require a microbial laminar hood and a roll of paper towels.

– The RSPM needs beakers for collecting discarded washing water; wash bottle of 95% ethanol; wash bottle of sterile distilled water; scalpels, forceps with curved and serrated tips; alcohol lamp for flame-sterilisation of steel instruments; glass plates (25 cm × 20 cm × 0.5 cm); cotton plugs; M1–100 CHL agar medium.

M1–100 CHL agar medium is composed of 1% malt extract; 2% agar; water in sufficient quantity to make 1 L; 100 mg chloramphenicol·L<sup>-1</sup> added to the molten medium after autoclaving.

– The SIM needs a commercial blender; a thermostatic incubator; a 0.5-cm screen; a series of three nested sieves (mesh-size: 1 mm, 50 µm and 25 µm) of 20 cm diameter; borosilicate glass bottles of 200 mL capacity with no-drip lips and screw-caps; 100-mL sterile flasks; beakers of 250 mL capacity; magnetic bars; magnetic stir plate; a basin; a wash bottle of distilled water; 90-mm sterile polystyrene petri dishes; PBB agar medium [1].

PBB agar medium is composed of 15 g glucose; 0.5 g yeast extract; 0.5 g KNO<sub>3</sub>; 1 g

$\text{KH}_2\text{PO}_4$ ; 0.5 g  $\text{MgSO}_4$ , 7  $\text{H}_2\text{O}$ ; 2 g agar; water in sufficient quantity to make 1 L. After autoclaving of the PBB agar medium, 100 mg chloramphenicol·L<sup>-1</sup> + 40 mg chlortetracycline·L<sup>-1</sup> + 1 mL Tergitol NP10 (nonyl phenyl polyethylene glycol ether with ethylene oxide)·L<sup>-1</sup> + 1 mg thiabendazole·L<sup>-1</sup> of PBB medium are added. Before incorporation into PBB medium, the two antibiotics (chloramphenicol and chlortetracycline) must be simultaneously dissolved in 5 mL of 95% ethanol.

## Protocol

### Root slice plating method

- Step 1

Prepare and select necrotic root subsamples: using a water nozzle, thoroughly wash the root sample; dry the root surfaces with a paper towel; from primary or secondary roots, cut off thirty or more 4–5-cm long root fragments bearing reddish to black necrotic lesions that are currently attributed to nematodes; select fragments with lesions ranging from individual flecks to large girdling necrosis.

*Note:* fragments showing obvious physical damage or extensive rot must be discarded to avoid fungal contamination during subsequent isolations.

- Step 2

Produce the fungal isolation under the laminar hood: flame-sterilise the glass plate; while handling a root fragment with steel forceps, wipe it gently with a 95% ethanol-soaked cotton plug; rinse the root fragment over a beaker with a jet of distilled water from the wash bottle and leave it to dry on a triple thickness of clean paper towels; do the same for the other selected root fragments.

*Note:* disinfect the glass plate with ethanol as frequently as possible between fragment disinfection operations.

Flame-sterilise the glass plate again; set five disinfected root fragments regularly along the length of the glass plate; using a flame-sterilised scalpel, cut a 3–4-mm thick slice at the junction between the healthy and necrosed tissue; with flame-sterilised forceps, place a slice standing on its edge at the end of the plate; using two scalpels alter-

nately (one working, the other cooling after flame-sterilisation), do the same for the other four fragments.

Flame-sterilise the forceps again and then plate the five root slices on the same petri dish of M1–100 CHL medium; repeat the operation for the other root fragments, while still processing them by groups of five in petri dishes of M1–100 CHL medium.

Incubate isolation medium dishes at room temperature (25 °C) for 4–6 days.

- Step 3

Determine *Cylindrocladium* spp. isolation frequency: count *Cylindrocladium* colonies as well as other fungal colonies. The fraction [(number of *Cylindrocladium* colonies) / (number of root slices)] gives an estimation of the root isolation frequency of *Cylindrocladium* fungi in the plot, while [(number of *Cylindrocladium* colonies) / (total number of isolated fungal colonies)] gives an estimation of *Cylindrocladium* fungi within the mycoflora associated with root lesions.

### Soil isolation method

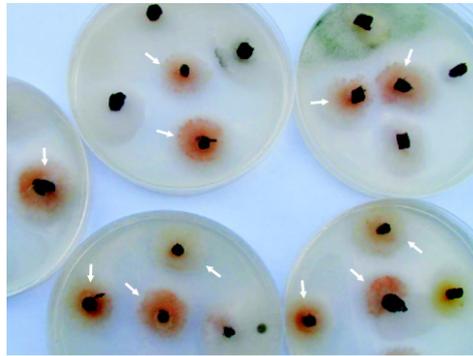
- Step 4

Preparation of soil sample: sieve the fresh soil sample through a 0.5-cm screen; reserve some 80-g soil subsamples to measure dry weight (DW) at 105 °C (drying to constant weight); place another 80-g soil subsample with 200 mL of tap water in a commercial blender and blend at low speed for 20 s; allow to settle for 20 s and blend again for 20 s; pour the resulting soil + debris solution through a series of 1-mm, 50- $\mu\text{m}$  and 25- $\mu\text{m}$  nested sieves; using a nozzle connected to a water tap, wash the 1-mm sieve at the top of the column for 1 min; remove it and discard its contents; wash the 50- $\mu\text{m}$  sieve above the 25- $\mu\text{m}$  sieve for 2 min.

*Note:* to avoid sieve obstruction, it is essential to continuously move the residues around with the water jet while gently shaking the sieve column.

Place the 50- $\mu\text{m}$  sieve in a basin with a 1 g·L<sup>-1</sup>  $\text{CaClO}_2$  solution so that the solution is just covering the residues in the sieve; incubate for only 10 s; remove the sieve from the basin and rinse its contents with the nozzle for 2 min; using a wash bottle of distilled water, collect the disinfected residues in a

**Figure 1.**  
*Cylindrocladium* colonies obtained from banana root lesions cultured on M1-100 CHL agar medium plates, using the Root Slice Plating Method.



**Figure 2.**  
Chains of chlamydospores and brown microsclerotia of a *Cylindrocladium* colony grown on a M1-100 CHL agar medium plate.



**Figure 3.**  
Conidiophore of a *Cylindrocladium* fungus showing a globose vesicle and cylindrical conidia.



flask and adjust the volume to 100 mL with sterile distilled water (flask No. 1); repeat this procedure with the 25- $\mu$ m sieve and finally collect its disinfected contents in a

second flask adjusted to 100 mL (flask No. 2).

• Step 5

Prepare the medium and seed the filled petri dishes: prepare 1 L of PBB agar medium and autoclave it for 15 min at 120 °C; make 100-mL aliquots of molten PBB medium in borosilicate glass bottles; maintain two of these bottles at 48 °C in a thermostatic incubator, while storing those that are solidifying for other future soil isolations.

Under the laminar hood, add the required quantities of antibiotics (chloramphenicol and chlortetracycline), Tergitol NP10 and thiabendazole to the two 100-mL molten PBB medium bottles; gently shake and put the amended medium bottles back in the incubator at 48 °C.

Under the laminar hood, fill a sterile beaker with the contents of flask No.1; add a magnetic bar; while stirring on a magnetic plate, remove  $x$  mL [ $x$  ranging from (3 to 6) mL] and add them to the first completed 100-mL PBB medium bottle; gently shake and pour before solidification into a dozen empty sterile petri dishes.

*Note:* fill petri dishes so that the agar layer is as thin as possible.

Do the same with flask No. 2 and the second 100-mL-PBB medium bottle [quantity  $x'$ ,  $x'$  ranging from (3 to 6) mL].

Incubate the petri dishes at room temperature (25 °C) for 8 days during which *Cylindrocladium* colonies will appear if present.

• Step 6

Calculate the inoculum density as follows: if  $c$  is the number of *Cylindrocladium* colonies found in the series of PBB medium dishes corresponding to flask No. 1, and  $c'$  the number found in dishes from flask No. 2, the inoculum density [ID, expressed in colony-forming units (cfu) per g of dried weight (dw)] in the soil of the banana plot is estimated by the formula:  $(100 / dw) \times [(c / x) + (c' / x')]$ .

**Troubleshooting**

Two main problems can occur:

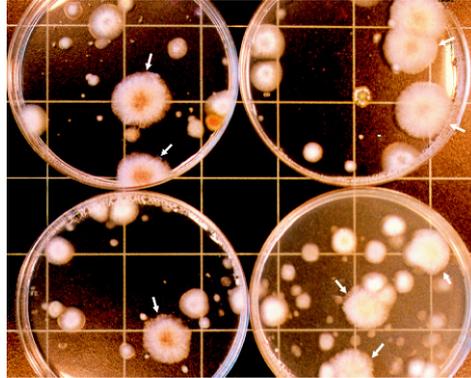
(a) For the SIM, there is abundance of bacterial contaminants in dishes due to deeply

rotten root fragments and/or cutting root slices directly in necrosed tissue.

*Solution:* increase the chloramphenicol concentration to (150 or 200) mg·L<sup>-1</sup> and/or cut root slices just at the limit between the healthy and necrosed tissue.

(b) For the SIM, there is obstruction of sieves by soil particles due to too static a wet-sieving procedure.

*Solution:* when spraying water in the sieves, make sure to actively move residues with the water jet while simultaneously shaking the series of nested sieves.



**Figure 4.** *Cylindrocladium* colonies obtained from soil isolation in PBB agar medium plates.

### 3. Typical results obtained

When using the RSPM, typical *Cylindrocladium* colonies are isolated in petri dishes (*figure 1*). When young, these colonies are yellowish on M1–100 CHL medium and, after a few days, they become tan to brown. Quite early, they produce numerous chlamydospores and microsclerotia (*figure 2*). Their conidiophores bear a vesicle-tipped stipe and cylindrical conidia (*figure 3*).

When using the SIM, typical *Cylindrocladium* colonies are isolated in petri dishes with the PBB medium (*figure 4*). The colonies originate from microsclerotia embedded or not in banana root tissue. The inoc-

ulum density is known to range from (0 to 40) cfu in naturally infested banana fields.

### References

- [1] Phipps P.M., Beute M.K., Barker K.R., An elutriation method for quantitative isolation of *Cylindrocladium crotalariae* microsclerotia from peanut field soil, *Phytopathol.* 66 (1976) 1255–1259.
- [2] Risède J.-M., Spatial and temporal distribution in soils of *Cylindrocladium* sp. fungal pathogen of bananas, *Phytopathol.* 85 (1995) 1563 (abstract).
- [3] Crous P.W., Taxonomy and pathology of *Cylindrocladium* (*Calonectria*) and allied genera, *Am. Phytopathol. Soc. (APS Press)*, St. Paul, USA, 2002, 294 p.