Long-term maintenance of *Cylindrocladium* strains and procedures for inoculum production

Jean-Michel Risède*, Béatrice Rhino

CIRAD-Persyst, UPR Systèmes bananes et ananas, Station de Neufchâteau, Sainte-Marie, 97130 Capesterre-Belle-Eau, Guadeloupe

jean-michel.risede@cirad.fr, beatrice.rhino@cirad.fr

Long-term maintenance of *Cylindrocladium* strains and procedures for inoculum production.

Abstract — Introduction. The techniques presented allow a long-term conservation of *Cylindrocladium* strains to preserve them from major morphological and physiological changes. The principle of the methods applied, key advantages, starting plant material, time required and expected results are presented. **Materials and methods**. The necessary laboratory materials, and details of the nine steps achieved for the monospore purification of *Cylindrocladium* strains, their long-term maintenance, and the production of conidia and microsclerotia are described. **Results**. When revived, purified strains generate actively growing colonies within 48–72 h. Such colonies can then be readily used for conidia or microsclerotia production.

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

Conservation à long terme des souches de *Cylindrocladium* et procédures pour la production d'inoculum.

Résumé — Introduction. Les techniques présentées permettent une conservation à long terme des souches de *Cylindrocladium* pour les préserver d'importants changements morphologiques et physiologiques. Le principe des méthodes appliquées, leurs principaux avantages, le matériel végétal nécessaire, le temps requis et les résultats attendus sont présentés. **Matériel et méthodes**. Le matériel de laboratoire nécessaire, ainsi que le détail des neuf étapes utilisées pour la purification monospore de souches de *Cylindrocladium*, leur conservation à long terme, et la production de conidies et de microsclerotes sont décrits. **Résultats**. Une fois réactivées, les souches purifiées produisent des colonies qui s'accroissent activement en 48–72 h. De telles colonies peuvent alors être aisément utilisées pour la production de conidies ou de microsclerotes.

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

* *Correspondence and reprints*

1. Introduction

Application

The techniques presented allow a long-term conservation of *Cylindrocladium* strains to preserve them from major morphological and physiological changes. The original characteristics of strains are thereby conserved, so they are suitable for use in further research studies (pathogenicity, taxonomy, etc.). Inoculum production is fully addressed for pathogenicity tests where large quantities of laboratory-grown conidia or microsclerotia are required, particularly for soil infestation assays. All of these techniques were initially intended for *Cylindrocladium* species issued from banana cropping systems, but were afterwords successfully extended to different *Cylindrocladium* strains, irrespective of the species or the originating host plant.

Fruits, 2008, vol. 63, p. 193–197

DOI: 10.1051/fruits:2008011
www.fruits-journal.org

Principle

Single conidium isolation of *Cylindrocladium* strains recently obtained from plant or soil samples [1] is first carried out to ensure purity. Purified isolates are then grown on sterilised banana leaf fragments from banana leaf agar (BLA) medium and further stored by conservation in 10% glycerol at – 80 °C. The basal metabolism is thus reduced. The conserved *Cylindrocladium* strains are revived on M2 (see the composition below) agar medium when needed.

Heavy conidiogenesis is induced by subculturing strains on leaf fragments from BLA medium under continuous lights. Conidia are harvested by shaking leaf fragments with intense sporulation in a Triton X–100 solution and then filtering the resulting suspension. The final conidial concentration can then be measured using a Malassez hemacytometer.

Heavy microsclerotia production is induced by inoculating M2 agar medium plates with a suspension of mycelial fragments. Cultures are then incubated in the dark for 7 weeks. Microsclerotia are extracted using a combination of blending, wet sieving and decanting procedures (derived from [2]).

Key advantages

The long-term maintenance procedure is easy and suitable for maintaining stability and viability of *Cylindrocladium* cultures without requiring liquid nitrogen storage and related expensive apparatus.

The conidia production technique is efficient, even for *Cylindrocladium* strains for which conidiogenesis is sparse on current culture media. In tropical countries, BLA medium can be more appropriate than carnation leaf agar (CLA) medium, which was initially proposed by Fisher *et al.* [3] for preserving *Fusarium* species and was further extended to *Cylindrocladium* species by Crous *et al.* [4]. Carnations are rare in these countries, while bananas are common.

The microsclerotia production technique is easy to process and efficient.

Starting material

The techniques require *Cylindrocladium* strains recently isolated from diseased tissue or soil [1], and 2-month-old weaned micropropagated banana plantlets that have never been sprayed with fungicides.

Time required

– From single conidium purification to freezing at – 80 °C, approximately 3 weeks are necessary.
– The experimental work lasts 4–5 h.
– Complete experiment for conidia production requires 8 d.
– Experimental work before and after plate incubation takes 2 h.
– To complete the entire procedure for microsclerotia production, including plate incubation time, approximately 7 weeks are necessary.
– Experimental work before and after extraction takes 6–7 h.

Expected results

– For the long-term maintenance procedure, conservation on banana leaf fragments dipped in 10% glycerol has allowed our lab to successfully store *Cylindrocladium* strains for at least 6 years without subculturing; probably a longer conservation time is possible.
– The yield of the conidial production technique is variable with strains.
– The microsclerotial production technique yields about, 7000–13000 microsclerotia after extraction from one multiplication medium plate.

2. Materials and methods

Laboratory materials

To achieve the protocols recommended, you need:

– a binocular stereomicroscope, a microbial laminar hood, freezers (– 20 °C and – 80 °C), an autoclave, a tube shaker, a centrifuge, a commercial blender, a lighted thermostatic

incubator (25 °C ± 2 °C) equipped with cool white and near-ultraviolet tubes;
– glass petri dishes, steel forceps with curved and serrated tips, micropipettes and tips, glass needles, Lazy-L polypropylene spreaders;
– sterile distilled water, 2% water-agar (w/v) plates, plates with M2 agar medium (2% malt extract; 2% agar; water: q.s.p. 1 L), 10% glycerol;
– polypropylene cryogenic vials (1 mL) and corresponding racks, scalpels, a chronometer, 50-mL Pyrex tubes, 0.5-L beakers, 32-µm sieves, a Triton X-100 solution, a Malassez hemacytometer, 50-mL sterile conical polypropylene tubes, 2-mm glass marbles, Parafilm, series of three nested sieves with 500 µm, 200 µm and 80 µm mesh size, a 1-L graduated cylinder, a vacuum pump.

Protocols

Cylindrocladium monospore strain purification

• Step 1. Preparation of banana leaf agar medium dishes
– Harvest the last emitted unfurled leaf from micropropagated banana plantlets. Wipe these leaves gently with a distilled water-soaked cotton plug to externally clean them.
– Cut off (2 × 3) cm leaf rectangles. Put them in a glass petri dish and autoclave for 15 min at 120 °C. Let them cool.
– Under the laminar hood, using flame-sterilised forceps, plate 3–4 sterilised leaf fragments on the surface of previousely prepared water-agar dishes.

• Step 2. Induction of conidial sporulation of the *Cylindrocladium* strains
– With the *Cylindrocladium* strain to be purified, inoculate each of the BLA medium plates at 8–9 different locations, but always near a leaf fragment.
– Label plates and incubate them for 7 days at 25 °C in a thermostatic incubator under continuous fluorescent cool white and near-ultraviolet lights.

• Step 3. Single conidium isolation
– Place a 50-µL drop of sterile distilled water at the centre of a water-agar plate.
– With the tip of a sterile glass needle, gently wrap a heavy sporulated leaf fragment from a 7-day-old *Cylindrocladium* culture grown on BLA agar medium. Then dip it in the water drop at the centre of the water-agar plate.
– Streak the contaminated drop over the whole surface of the water-agar plate with a sterile Lazy-L spreader. Do this while turning the plate 360° to yield an even distribution of conidia on the agar surface. Do the same for all *Cylindrocladium* strains to be purified.
– Incubate plates at room temperature (25 °C) for 3–4 h to allow conidia germination.
– For each strain, under the stereomicroscope, select a well isolated germinating conidia in the inoculated plate. Using a fine-bladed scalpel, cut off an agar block that only supports the selected conidia and transfer it to a M2 medium plate. The agar block must be as small as possible, *i.e.,* about 4 mm^2 of area.
– Incubate M2 agar medium plates at 25 °C for 4–5 days. They constitute the purified *Cylindrocladium* cultures.

Long-term maintenance procedure for *Cylindrocladium* cultures

• Step 4. Preparation of strains before freezing
To transfer *Cylindrocladium* strains onto BLA medium, inoculate BLA agar medium plates with the purified *Cylindrocladium* strains and incubate them for 10 days at 25 °C in a thermostatic incubator under continuous fluorescent cool white and near-ultraviolet lights. *Note*: at the end of this step, leaf fragments generally show both conidia and microsclerotia.

• Step 5. Conservation of cultures at – 80 °C
– For each strain, fill four 1-mL cryogenic vials with 700 µL 10% glycerol; screw caps loosely and autoclave for 15 min at 120 °C; tighten caps and cool vials under the laminar flow.
– In each 10-day-old *Cylindrocladium* culture grown on BLA medium, select three well sporulated leaf fragments. Cut them with two flame-sterilised scalpels in the lid of the corresponding plates. Distribute the

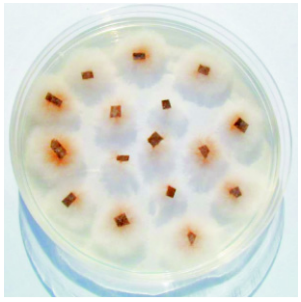

Figure 1.
Typical 3-day-old *Cylindrocladium* colonies yielded by pre-colonised banana leaf fragments that were stored in 10% glycerol at – 80 °C.

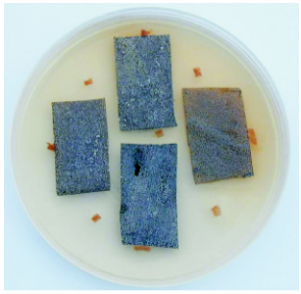

Figure 2.
Sporulated culture of a *Cylindrocladium* strain on banana leaf agar medium. After incubation (1 week) under continuous cool white and near-ultraviolet lights, leaf fragments are covered with a white dust of conidia.

Figure 3.
Clusters of *Cylindrocladium* microsclerotia embedded in M2 (2% malt extract; 2% agar; water: q.s.p. 1 L) agar medium that was seeded 7 weeks earlier with a suspension of mycelial fragments.

obtained small leaf pieces equally in the four prepared cryogenic vials. Label the vials and put them in their freezing racks. Let them equilibrate at room temperature for 1 h.
– Transfer racks into a – 20 °C freezer for 12 min. Then transfer them immediately into a neighbouring – 80 °C freezer.

• Step 6. Retrieving of strains
– Thaw the vials at room temperature (25 °C) for 60–90 min.
– Pick up leaf pieces with a flame-sterilised scalpel and transfer them to M2 agar medium plates to reinitiate actively growing *Cylindrocladium* cultures.

Conidia and microsclerotia production technique

• Step 7. Technique for production of conidia
– Prepare a heavily sporulated *Cylindrocladium* culture on BLA medium as described in step 2.
– Select heavily sporulated fragments under the stereomicroscope.
– Prepare a 250 $\mu L \cdot L^{-1}$ Triton X–100 solution. Fill some 50-mL Pyrex tubes with 15 mL of this solution. Put 3–5 leaf fragments in each tube.
– Shake tubes at 1 600 rpm for 20 s and pour the contents into a 32-µm sieve over a 0.5-L beaker. Rinse each tube twice with 7.5 mL of the Triton X–100 solution and filter this rinsing solution through the 32-µm sieve.
– Concentrate the conidial suspension collected in the beaker by centrifugation at 2 000 rpm for 2 min. The number of conidia per mL can then be determined with a Malassez hemacytometer before adjustment and use.

• Step 8. Technique for mass production of microsclerotia
– Under the laminar hood, inoculate a purified *Cylindrocladium* strain at 5–6 locations on M2 agar medium plates. Incubate for 3 days at 25 °C.
– Fill 50-mL sterile conical polypropylene tubes with approximately 3 mL of 2-mm glass marbles and 5 mL of sterile distilled water.
– Using a flame-sterilised scalpel, cut each colony formed on agar medium in small, approximately 2-mm^2 blocks. Aseptically transfer the blocks from four colonies to each tube containing distilled water and marbles. Shake at 2 500 rpm for 2 min to obtain a mycelial suspension.
– Pour the mycelial suspension from the different tubes into a single sterile 500-µm sieve over a sterile 0.5-L beaker. Rinse each tube over the sieve with approximately 30 mL of sterile distilled water. The resulting sieved suspension can be further concentrated by centrifugation at 2,000 rpm for 2 min.
– Inoculate M2 agar medium plates with 130 µL of sieved mycelial suspension. Streak a drop of suspension over the whole surface of the agar medium plate using a sterile Lazy-L spreader. Do this while turning the plate 360° to obtain an even distribution of mycelial fragments on the agar medium surface.
– Seal plates with Parafilm and incubate in darkness at 25 °C for 7 weeks.

• Step 9. Extraction of microsclerotia
– Place 20 to 25 of the above microsclerotia produced cultures in a commercial blender. Just cover them with tap water. Blend at high speed for 20 s and allow to settle for 20 s. Repeat this blending-settling operation twice more.
– Pour the resulting suspension through a series of 500-µm, 200-µm and 80-µm nested sieves.
– Using a nozzle connected to a water tap, wash the 500-µm sieve over the rest of the column for 2 min. Remove the 500-µm sieve and discard its contents.
– Wash the 200-µm sieve in the same way over the 80-µm sieve. Discard its contents.
– Finally, wash the 80-µm sieve for 2-min but save its contents.
– Fill the bottom of a 50-mL conical polypropylene tube with approximately 1–2 mL of 2-mm glass marbles. Using a wash bottle of water, collect the contents of the 80-µm sieve by pouring it in the tube. Adjust the volume to 30 mL with water and shake at 2 000 rpm for 3 min. *Note*: this operation is designed to mechanically remove mycelial hyphae from the microsclerotia.
– Pour the suspension again through the series of 500-µm, 200-µm and 80-µm nested sieves. Quickly wash the first two sieves and

discard their contents. Wash the 80-µm sieve for 2 min and pour its contents into a 1-L graduated cylinder.
– Adjust the volume of the graduated cylinder to 1 L with tap water. Shake and decant for 5 min. Using a vacuum pump, remove the supernatant by only leaving approximately 100 mL of suspension in the cylinder. Repeat this refining operation until the supernatant becomes clear and visually devoid of mycelial mats. A clean suspension of microsclerotia is ultimately obtained.
– Before use, calculate microsclerotia concentration by counting the number of microsclerotia present within a known aliquot of suspension under a stereomicroscope.

3. Typical results obtained

When retrieved, purified strains that were conserved at – 80 °C on banana leaf fragments in 10% glycerol generate actively growing colonies within 48–72 h (*figure 1*). Such colonies can then be readily used for the production of conidia or microsclerotia. Intense conidiogenesis is thus obtained when growing *Cylindrocladium* strains on banana leaf agar medium under continuous lights (*figure 2*). *Figure 3* illustrates laboratory-produced microsclerotia just before extraction, while *figure 4* shows the microsclerotia suspension obtained after extraction.

References

[1] Risède J.-M., Isolation of *Cylindrocladium* spp. in roots and soils from banana cropping systems, Fruits 63 (2008) 57–61.

[2] Risède J-M., Éléments de caractérisation de *Cylindrocladium* sp. agent de nécroses racinaires du bananier en Martinique, Fruits 49 (1994) 167–178.

[3] Fisher N.L., Burgess L.W., Toussoun T.A., Nelson P.E., Carnation leaves as a substrate and for preserving cultures of *Fusarium* species, Phytopathology 72 (1982) 151–153.

[4] Crous P.W., Philipps A.J.L., Wingfield M.J., Effects of cultural conditions on vesicle and conidium morphology in species of *Cylindrocladium* and *Cylindrocladiella*, Mycologia 84 (1992) 497–504.

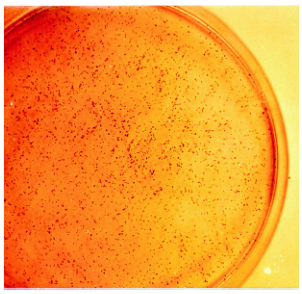

Figure 4.
Suspension of microsclerotia from a *Cylindrocladium* strain. These microsclerotia were produced and extracted by the method described.