# Bean Pathogens: Practical Guide for Lab and Greenhouse Work

CIAT
International Center for Tropical Agriculture
*Since 1967 / **Science to cultivate change***

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# General Contents

# Acknowledgements

These *Laboratory Practice Guides* are the result of work conducted in CIAT's Bean Pathology Laboratory since its beginnings in the 1970s. They bring together the practical experience acquired by its authors over the last 20 years.

Many people have helped strengthen the area, contributing knowledge that, today, translates into the publication of this laboratory practice manual on managing bean pathogens. In this long list of people, the following stand out for their scientific contributions to these *Guides*: the bacteriologists Ángela Tascón (R.I.P.), Belén Vesga, and Dr Lucía Afanador; and the plant pathologists Drs Fernando Correa, Elizabeth Álvarez, Marcial Antonio Pastor-Corrales, and George Mahuku, who were, in their time, leaders in the field of bean pathology.

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# Introduction

This manual brings together nine *Laboratory Guides* that describe protocols used by CIAT's Bean Pathology Laboratory. The *Guides* were conceived as reference or learning materials for researchers, students, and technicians who need to know how to manage, in the laboratory, eight important fungal and bacterial pathogens affecting the bean crop. Although bean-plant pathogens in the laboratory are subject to numerous procedures depending on research goals, the *Guides* focus on sample collection; isolation of pathogens; their multiplication; inocula preparation; conservation, storage, and recovery; and DNA extraction.

The empirical knowledge expressed within these *Guides* should be valuable to those who are scientifically interested in these microorganisms. Often laboratory guidelines do not exist, and researchers must rely on the expert knowledge of technicians who may not always be available because, for example, they are working on project backlogs. To help researchers and students learn the procedures and their application, these have been described in a step-by-step fashion, without scrimping on details.

The efficiency and utility of the procedures presented in the *Guides* have been tested and proven in the Bean Pathology Laboratory for more than 25 years. They are therefore effective in managing a pathogen and for bean germplasm screening using artificial inoculation methods (e.g., for resistance, tolerance, or susceptibility). They can also be used in other areas such as population studies of a pathogen. These two applications in particular are significant pillars in crop improvement programs.

We also hope that these *Guides* can be applied to similar pathogens affecting crops other than beans, thereby helping to strengthen agricultural research and contributing much towards improving crop productivity.

These documents are conceived as a dynamic guide where our readers or researchers could suggest the addition of new methodologies available that were not described here. So, please share your findings with the *Guides*' authors and help us to improve the content. This way we can all improve the Guides to the benefit of the scientific community. Thanks to the flexibility of updating offered by online publication, all such contributions will receive due recognition in the manual.

Thus we hope that, in time, these *Guides* will become an interactive site that collects the science and experience of many plant pathologists and laboratory workers across the world. We also hope that CIAT and other research centers will see the need to translate the *Guides* into languages other than English and Spanish. This would ensure the *Guides*' greater dissemination and thus the adoption of more effective laboratory practices throughout the world.

# Practical Guide 1

*Phoma exigua* var. *diversispora*

Ascochyta blight

# Managing the fungus in the laboratory

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Bean Plant Pathology

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## Contents

# Considerations

Photo I–1

Photo I–2

The fungus *Phoma exigua* var. *diversispora* (Bubák) Boerema causes the bean disease known as ascochyta blight, so called after its old taxonomic name. Optimal environmental conditions for the development of the disease are temperatures between 15 and 20 °C and relative humidity between 80 and 100%. Such conditions are found at altitudes of more than 1500 m above sea level.

Initial symptoms appear on leaves as dark grey circular lesions (**Photo I–1**) that, as they grow, appear as groups of concentric circles. In these, masses of small black pycnidia develop.

These lesions appear later on petioles, peduncles, pods, and stems. If environmental conditions are favorable, attack from this pathogen appears as severe foliage burning, which leads to premature defoliation and, eventually, plant death.

The fungus is transmitted by contaminated seeds. **Photo I–2** shows typical blight lesions on pods.

# Procedures

## A. Collecting and shipping samples

To isolate the fungus *Phoma exigua*, first collect bean tissues exhibiting typical symptoms of the disease and well-developed lesions.

Never collect old or senescent plant tissues, as these carry saprophytes that would complicate the isolation of *P. exigua*. Discard all diseased plant tissues with signs of insect damage or symptoms of other diseases. Also take into account that:

- The isolation of this pathogen is easier from diseased leaf tissue, as different kinds of lesions appear on leaves. From each lesion, several strains can also be obtained.
- When collecting diseased pods, prefer green ones because procedures are easier to carry out on these.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying samples
- Marker pen and fieldbook

- ***Paper towels.*** Wrap collected samples of diseased bean tissue, whether of pods, leaves, or stems, in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples of diseased tissue, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach the labels to their corresponding samples.

## *Recommendations*

- Indicate on the label if collection was in a farmer's field or on an experimental station.
- If there are not enough labels for all the samples, code the bags and record the full information in a fieldbook.
- Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once in the laboratory, and if they are not to be immediately processed, leave them out to finish drying on paper towels.

## Processing steps

- ***Step 1***
  Take samples of leaf or pod tissues from plants presenting ascochyta blight (***see*** *I.A.*).

- ***Step 2***
  Wrap each sample in a paper towel, place it in a paper bag or envelope, and firmly attach the corresponding label (***see*** *I.A.)*.

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons explained above.

## B. Preparing PDA culture medium

The letters in the abbreviation "PDA" correspond to components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- PDA powder (39 g)
- 1 L distilled water
- Two 1000-mL erlenmeyer flasks
- 50 petri dishes
- Large receptacle (e.g., beaker)
- Autoclave
- Balance

### Preparation

- Weigh the ingredients, place them in a large receptacle such as a beaker, and add distilled water. Pour 500 mL of the resulting solution (PDA culture medium) into each erlenmeyer flask.
- Autoclave the flasks containing the medium for 40 min at 121 °C and a pressure of 20 psi until sterilized.
- Leave the sterilized medium until cool enough to pour into petri dishes at 20 mL per dish.

***Note:*** The protocol for growing the fungus *P. exigua* in PDA culture medium is simple because plant tissue colonized by the pathogen usually harbors few or no sacrophytes, thus facilitating isolation.

## C. Isolating *P. exigua* var. *diversispora*

### *1. On PDA culture medium*

#### Materials

- Samples of diseased plant tissues
- Petri dishes containing PDA culture medium
- 60- and 100-mm petri dishes
- Laminar-flow chamber
- 2.5% sodium hypochlorite
- Incubator
- Needle
- Scissors
- Bunsen burner
- Paper towels
- Stereoscope
- Pipettes
- Tweezers
- Sterilized distilled water
- Bent-glass streaking rod

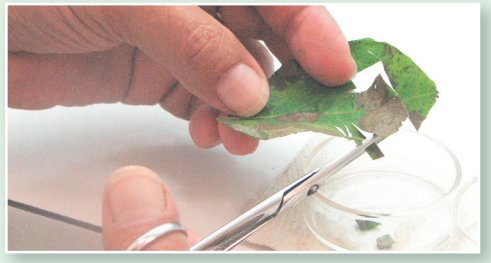


Photo I–3

Photo I–4

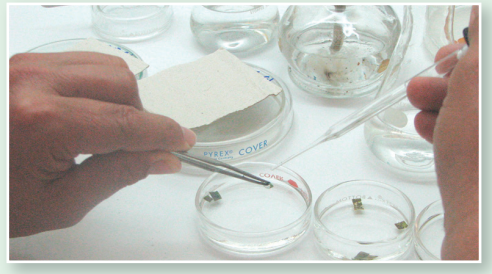


Photo I–5

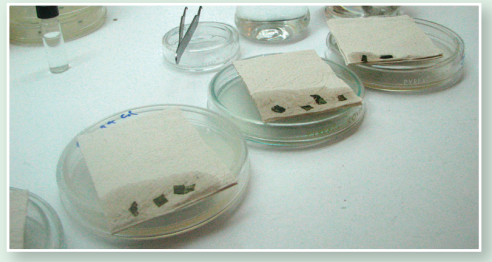


Photo I–6

***Note*:** All procedures should be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

## Processing steps

- ***Step 1***
  Use sterilized scissors to cut a sample of diseased tissue into several pieces (**Photo I–3**).

- ***Step 2***
  Disinfect the pieces by placing them in a 60-mm petri dish containing a solution of 2.5% sodium hypochlorite. Leave for 3 min (**Photo I–4**). Take up another petri dish and use a sterilized pipette to rinse the disinfected pieces with sterilized distilled water (**Photo I–5**). Then place the disinfected and washed pieces on sterilized paper towels to dry for 10 min (**Photo I–6**).

- ***Step 3***
  Once the pieces are dry, use sterilized tweezers to select 4 pieces and transfer them to a petri dish containing PDA culture medium (**Photo I–7**). Proceed likewise for other petri dishes.

- ***Step 4***
  Incubate the dishes at 20 °C for 10 days, by which time the fungus should be producing mycelium, pycnidia, and conidial masses. Use the last-mentioned structures to generate monosporic isolates (**Photo I–8**) as described in the next section.

## 2. As a monosporic culture

Because a sample of diseased tissue may carry a mixture of pathogenic strains, the genetic purity of a *P. exigua* isolate is assured only when that isolate is generated from a single conidium.

### Processing steps

- ***Step 1***
  Use a stereoscope to select a conidial mass from the fungus. With a flamed-sterilized needle, very gently touch the mass so that several conidia will adhere to the needle point (**Photo I–9**).

Photo I–7

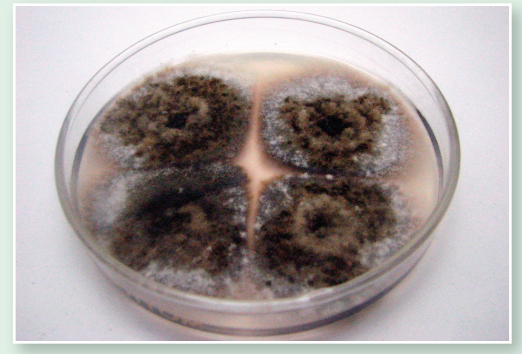


Photo I–8

Photo I–9

Photo I–10

- ***Step 2***
  Over PDA culture medium in a petri dish, remove the conidia from the needle with sterilized distilled water. That is, draw up the water in a pipette and deposit 4 to 6 drops onto the needle held over the medium (**Photo I–10**). Spread the conidia-containing drops evenly across the medium's surface, using a bent-glass streaking rod.

  Ensure that the streaking rod is flame-sterilized before each use to prevent contaminating the medium.

- ***Step 3***
  Incubate the petri dishes at 20 °C for 24 h, by which time, the conidia should be germinating.

- ***Step 4***
  Examine the germinated conidia under a stereoscope, select one that is isolated from others, and remove it with a flamed-sterilized needle, transferring it to a petri dish containing PDA culture medium.

- ***Step 5***
  Incubate the dish at 20 °C. The conidium should develop so that, after 12 days, it completely covers the medium with the structures of a monosporic fungus.

  The monosporic isolate is used to assure the genetic purity of the strains which is required for diverse procedures such as inoculation, DNA extraction, and conservation.

## D. Increasing *P. exigua* on PDA medium with added bean leaves

Increasing must be carried out with a monosporic isolate in a petri dish containing PDA medium (*see above, I.C.2.*). For some strains, adding sterilized bean leaves to the medium will stimulate the fungus to sporulate.

### Preparing the leaves

Select only those bean leaves that are free of fungicides, are mature leaves at 30 days old, and are medium sized. Note that young leaves disintegrate when autoclaved, and very large leaves will not fit into petri dishes. Sterilize the selected leaves by autoclaving.

Place about 20 leaves, with the lower surface uppermost, in large petri dishes and spray with distilled water. The dishes are then wrapped, first, in aluminum foil, and then in wrapping paper, and autoclaved.

## Materials

- Healthy sterilized bean leaves, free of fungicides
- Petri dishes containing PDA culture medium
- Fungal isolate cultured on PDA medium
- Bunsen burner
- Sterilized distilled water
- Tweezers
- Bulb pipette
- Incubator
- Bent-glass streaking rod

Photo I–11

## Processing steps

- ***Step 1***
  Use sterilized tweezers to transfer 1 or 2 sterilized bean leaves to a fresh dish containing PDA culture medium (**Photo I–11**).

- ***Step 2***
  Meanwhile, use a mini glass pipette to add a little sterilized distilled water to the monosporic isolate. With that same pipette, scrape the surface of the fungal growth to detach conidia and mycelium, thus obtaining a suspension.

- ***Step 3***
  Deposit a little of the fungal suspension on the bean leaves described in *Step 1*. Spread the suspension over the leaf's entire surface, using a flame-sterilized, bent-glass, streaking rod.

- ***Step 4***
  Incubate the petri dishes at 20 °C for 12 days, by which time, the fungus will have produced sufficient conidia for inoculating plants in the greenhouse (**Photo I–12**).

## E. Inoculating plants

### *1. Inoculum for use in the greenhouse*

#### Processing steps

- ***Step 1***
  Ten days before inoculation, increase the fungus according to the number of plants to be inoculated. Calculate the number of petri dishes containing PDA medium needed to make the required amount of inoculum. Then grow the fungus, as described above in ***I.D.***

  A petri dish carrying abundant sporulation would produce about 1 L of inoculum, which would be sufficient for about 120 pots at 3 plants per pot. Calculation, however, depends on the isolate's characteristics, as not all isolates sporulate the same way.

Photo I–12

Photo I–13

- ***Step 2***
  Add sterilized distilled water to the fungal growths. Then scrape the surface with a sterilized spatula to detach and resuspend the conidia in the water. Filter the conidial suspension through sterilized gauze to separate solid particles such as agar residues and mycelium. Collect the filtrate containing conidia in a sterilized medium-sized beaker or large sterilized petri dish (**Photo I–13**).

  The filtration will facilitate the conidial count and also prevent the nozzle of the DeVilbiss nebulizer from blocking. The nebulizer is used, together with a compressor, to apply inoculum to the plants. An airbrush or liquid atomizer can be used instead of a DeVilbiss.

- ***Step 3***
  Use a microscope to count the number of conidia found within the central grid of a hemocytometer, itself comprising one central and four outer squares. Multiply the number of conidia counted by 50,000. In this case, inoculum concentration should be $1 \times 10^6$ conidia per milliliter. Given the final volume of inoculum to be sprayed, and given the conidial concentration of the original inoculum, use the following formula to calculate the volume of the original inoculum needed for spraying:

  $$V_1 \times C_1 = V_2 \times C_2$$

For example, if 150 conidia are counted in the hemocytometer, then:

$$150 \times 50{,}000 = 7{,}500{,}000 = 7.5 \times 10^6$$
(conidia in 1 mL)

This is the concentration of the original inoculum (i.e., the filtrate in *Step 2*). If 250 mL of inoculum with a concentration of $1 \times 10^6$ conidia per milliliter are needed, then, by substituting these data in the previous formula, the value of the volume of the original (also called *initial*) inoculum needed (i.e., $V_1$) can be found, that is:

$$V_1 \times 7.5 \times 10^6 = 250 \text{ mL} \times 1 \times 10^6$$

Thus,

$$V_1 = \frac{250 \text{ mL} \times 1 \times 10^6}{7.5 \times 10^6} = 33.3 \text{ mL}$$

To obtain the final concentration desired, take 33.3 mL of the initial volume and adjust it to 250 mL with sterilized distilled water.

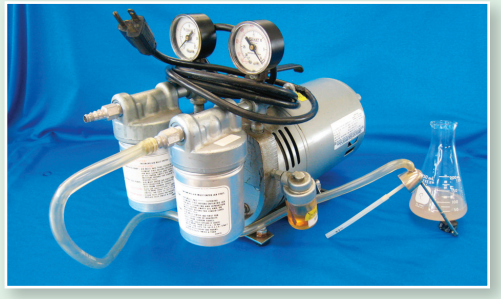


Photo I–14

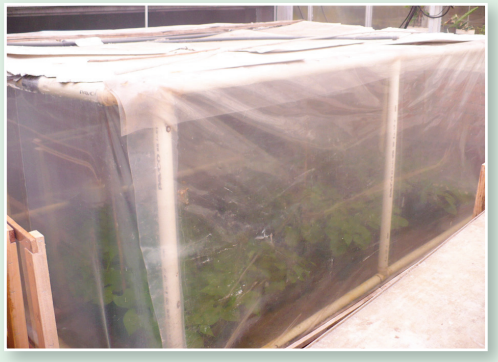


Photo I–15


Photo I–16

- ***Step 4***
  Once having the inoculum at the required conidia concentration, pour a volume of the suspension into a 250-mL erlenmeyer flask. Connect the flask to a DeVilbiss nebulizer (or airbrush) and this to a compressor (**Photo I–14**). Spray 17 day-old plants on their primary trifoliate leaf. The volume of inoculum applied to the leaf is calculated so that the foliar surface is left entirely wet without the inoculum draining or running off.

- ***Step 5***
  Place the inoculated plants in a humid chamber set at a relative humidity of more than 90% (**Photo I–15**). Leave the plants for 7 days. Then remove them from the chamber and place them on a table for another 3 days. Ten days after inoculation, evaluate the plants, using the **CIAT standard scale**,* with scores ranging from 1 to 9, where:

  1 to 3 indicate a resistant plant
  4 to 6 indicate a plant with an intermediate reaction
  7 to 9 indicate a susceptible plant

  **Photo I–16** shows a bean genotype that is susceptible to ascochyta blight. It scored 9 in an evaluation that used the scale described above.

* Available at: http://bit.ly/CIAT_STANDARD_SYSTEM_FOR_THE_EVALUATION_OF_BEAN_GERMPLASM

## 2. *Inoculum for use in the field*

### Materials

- Growths of *P. exigua* on PDA culture medium
- Industrial blender
- Hemocytometer
- Large receptacle
- Running water
- Funnel
- Spatula

### Processing steps

- ***Step 1***
  Pour destilled water into the bowl of an industrial blender and use a spatula to add fungal cultures and their PDA medium (**Photo I–17**).

  Fifty medium-sized petri dishes carrying 10-day-old fungus are sufficient to produce the inoculum needed to spray 1 hectare of beans. Calculate the quantity of water so that the mixture, when homogenized, is not too thick.

- ***Step 2***
  Blend the bowl's contents until a homogeneous suspension is obtained. Dilute it in 20 L of water.

Photo I–17

***Step 3***

Quantify the conidial concentration in the 20 L of homogeneous product, using a hemocytometer. Then calculate the volume of the final solution of inoculum according to the area of crop to be inoculated. Adjust the inoculum's final concentration. See *Step 4* below for an example of how to calculate for 1 ha.

- ***Step 4***

  Use a funnel to pour the inoculum into a receptacle that is easy to manage (**Photo I–18**). Firmly close the receptacle's mouth and constantly check for fissures or leaks.

  To artificially inoculate 1 ha of a bean crop in the field, as a minimum, 50 to 70 petri dishes containing well-sporulated growths of *P. exigua* are needed. Dilute the fungus in 140 L of water, thus obtaining a conidial suspension of the required concentration (1 x $10^6$ conidia per milliliter) for inoculating 1 ha of crop.

  Always carry out inoculations with this pathogen in the early evening when temperatures are dropping and relative humidity is increasing.


Photo I–18

# F. Conserving the fungus for storage

The fungus *P. exigua* can be conserved by lyophilization, on pieces of filter paper kept in glassine envelopes, or on pieces of filter paper impregnated with the fungus suspended in peptone-sucrose solution and kept in glassine envelopes.

## *1. Lyophilization*

This is the most reliable method for conserving microorganisms in storage.

### Materials (Photo I–19)

- Tree manifold lyophilizer with two types of support (rack and tree) and 48 ports for vials. Other models could be also used according to the provider's manual
- 0.5-mL neutral-glass vials for lyophilization, and rack
- Growths of fungus on PDA culture medium
- Distilled water
- Propane gas torch
- Long Pasteur pipettes
- Freezer
- Scissors
- Bunsen burner
- Filter paper
- Autoclave
- Marker pen and notebook
- Peptone at 10%
- Tweezers or inoculation needle

Photo I–19

Photo I–20

Photo I–21

Photo I–22

– Sucrose or dextrose at 20%
– Cotton wool
– Large receptacle

***Note:*** Fungal isolates should be prepared (see ***I.D.***) 12 days before lyophilization is carried out. The fungus should have sporulated well and be free of contaminants.

## Processing steps

- ***Step 1***
  Write in either small lettering or code on 1- to 2-cm squares of filter paper to identify the isolates to be lyophilized. Include the fungus's genus and species names, strain number (i.e., the code the laboratory uses), and storage date.

  If you write in code, write the full information in a notebook.

- ***Step 2***
  Use sterilized scissors to cut out the pieces of filter paper identifying each isolate and introduce each piece to the bottom of a vial.

- ***Step 3***
  Then prepare a cotton wool plug to stopper each vial so that it plugs the upper two thirds, as follows:

– Tease out a piece of cotton wool until it measures about 5 x 3 cm and roll it around a tweezers arm or needle of an inoculation needle, pressing between fingers and thumb until a plug is formed (**Photos I–20, I–21, and I–22**).

Leave a piece of unrolled cotton wool on the end opposite the plug.

– Introduce the plug into the vial and gently withdraw the tweezers arm or needle with one hand while holding the plug in the vial with the other.
– Prepare additional vials with their respective plugs to have spares on hand as needed. If a plug disintegrates on handling, it can therefore be replaced by one from the additional vials.

- ***Step 4***
  Autoclave all the vials with their respective plugs for 40 min to sterilize them.

- ***Step 5***
  **Preparing peptone and sucrose solutions.**
  Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a solution of either sucrose or dextrose at 20% by dissolving 20 g of the sugar in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix the solutions in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose (or peptone-dextrose) solution.

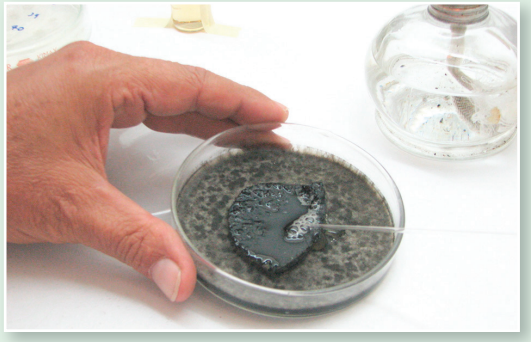


Photo I–23

Photo I–24

Photo I–25

- ***Step 6***
  Use a long Pasteur pipette to draw up 2 to 3 mL of the peptone-sucrose solution and deposit them on a fungal culture (**Photo I–23**). Homogenize the fungus within the solution by first scraping the culture (including mycelium and conidia) and then by repeatedly drawing up and expelling the mixture, using the same pipette.

- ***Step 7***
  Use the same pipette to draw up 2 to 3 mL of the homogenized suspension and deposit them inside a vial (**Photo I–24**), as follows:

  – In one hand take the pipette and, in the other, the vial with its respective plug as prepared in *Step 3* above.
  – Hold the pipette between the index finger and thumb and, using the little finger (or pinky) of the same hand, carefully withdraw the cotton wool plug from the vial by its loose end, keeping the plug firmly against the palm. Then deposit the fungal suspension in the pipette in the bottom of the vial, which should have the piece of filter paper identifying the strain.
  – Stopper the vial (**Photo I–25**) by gently introducing the plug, which had been held between the little finger and palm, back into it.

If the cotton wool plug comes into contact with another surface or other substance, it is contaminated. Discard it therefore and replace it by one of the additional plugs that had been prepared (see *Step 3* above) for such an eventuality.

- ***Step 8***
  Use flame-sterilized scissors to cut off that part of the plug remaining outside the vial (**Photo I–26**). Then use the point of a scissors arm to introduce the rest of the cotton wool plug into the vial until 1 cm is left between the lip of the vial and the plug (**Photo I–27**).

- ***Step 9***
  Place the vials in a rack. Then push the cotton wool plug to the bottom of each vial until it touches the piece of filter paper identifying the strain. To achieve this, use a long object such as an inoculation needle, constantly flame-sterilizing it to prevent contaminating the plugs (**Photo I–28**).

  The upper part of the vial, which has no plug, is sealed after lyophilization (see *Step 14* below).

- ***Step 10***
  Once the vials are organized, freeze them at 0 °C for 15 min. When the samples are frozen, lyophilization is initiated.


Photo I–26


Photo I–27


Photo I–28

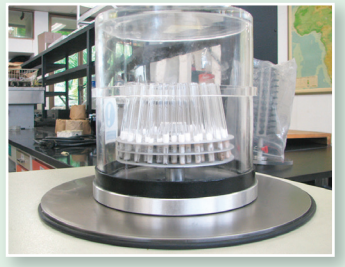


Photo I–29

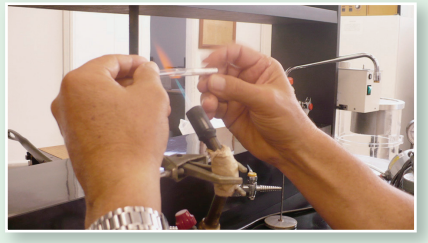


Photo I–30

Photo I–31

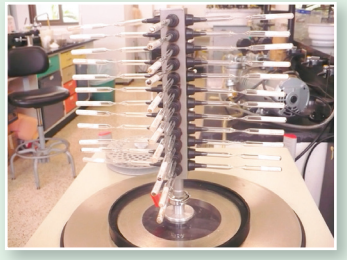


Photo I–32

- ***Step 11***
  Remove the rack from the freezer and place it inside the lyophilizer dome. Switch on the machine (**Photo I–29**). The temperature inside the apparatus will drop to -55 °C, at which point a vacuum pump will begin extracting water, drying the samples. This procedure takes 20 to 22 h to complete.

- ***Step 12***
  The next day, switch off the lyophilizer and remove the rack of vials. Prepare a propane gas torch for constricting the vials in the part opposite the sample. That is, make a neck in each vial by softening the glass with the torch's flame and pulling gently on the ends of the vial (**Photos I–30 and I–31**).

  This procedure needs considerable care to prevent burns to the hands. This step reduces the space by which air and therefore humidity can reach the sample before sealing is completed.

- ***Step 13***
  Once all the vials have been constricted, place them on the ports of the lyophilizer tree, inserting the open end into each port. Thus, the end carrying the sample remains visible (**Photo I–32**). Repeat the drying process in the lyophilizer for about 1 h to eliminate any humidity that may have reached the sample during *Step 12*.

- ***Step 14***
  Without switching the lyophilizer off, seal the vials, one by one, by vacuuming and then using the propane gas torch to melt the glass and thus constrict the neck (**Photo I–33**). Once the vials are sealed, lyophilization is completed.

***Note*:** If the vacuum is lost on sealing a vial, remove this part of the vial and replace it with a new one. Wait for the vacuum to reach the point of sealing before continuing to seal the other vials.

How long a lyophilized sample can last in storage has not been formally established. However, in our laboratory, lyophilized samples have been recovered after 30 years of storage. Lyophilized samples do have a disadvantage: to recover a sample, the entire vial must be broken. Several copies of each lyophilized sample must therefore be made to maintain an adequate collection.

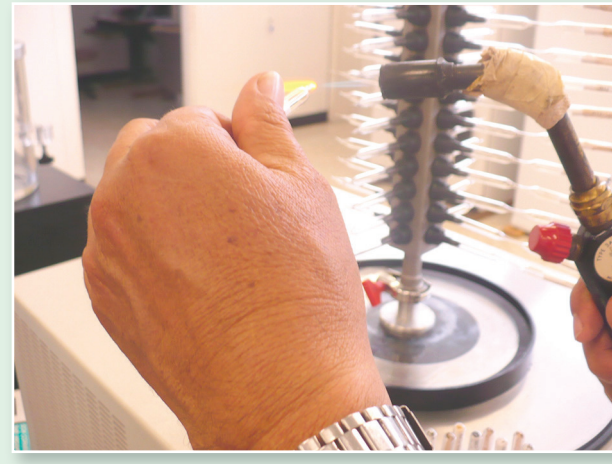


Photo I–33

## 2. Conservation as fungal growth on filter paper at -20 °C

This method permits long-term conservation of microorganisms.

### Materials

- Petri dishes containing PDA culture medium
- Sterilized filter paper
- Glassine envelopes
- Cultures of *P. exigua*
- Bunsen burner
- Tweezers
- Autoclave
- Sterilized petri dishes
- Incubator
- Sterilized distilled water
- Marker pen
- Pipette

### Processing steps

- ***Step 1***
  Cut the filter paper into 60 to 80 1-cm squares, place them in a petri dish or lidded container, and sterilize by autoclaving.

- ***Step 2***
  Place 10 of the squares in each petri dish containing PDA culture medium.

- ***Step 3***
  Prepare a suspension of conidia and mycelium of a fungal strain, as follows:

  – Deposit sterilized distilled water on a strain already grown in a petri dish.
  – Scrape the surface of the fungus with the pipette tip until a suspension is obtained.

- ***Step 4***
  Draw up a little of this suspension with the same pipette and place one drop on each square.

- ***Step 5***
  Incubate the petri dishes at 20 °C for 12 days.

- ***Step 6***
  After the 12 days, remove the squares of filter paper from the petri dishes, using sterilized tweezers and, complying with all conditions for asepsis and sterilization, deposit them in an empty and sterilized petri dish. Place the squares so that the fungus faces downwards. This should ensure that the papers will not curl on drying. Dry the papers at 24 °C for 7 days.

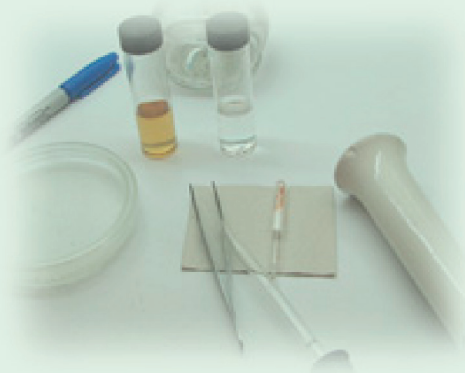


Photo I–34

Photo I–35

- ***Step 7***
  Use flame-sterilized tweezers to transfer the dried paper squares to small and sterilized glassine envelopes (**Photo I–34**). Introduce these into either a larger glassine bag or small box suitable for storage (**Photo I–35**). Write information on the fungus on this larger bag (or box), and store at -20 °C.

## 3. *Conservation as a suspension on filter paper at -20 °C*

### Materials

- Peptone-sucrose solution, prepared as described in *I.F.1, Step 5*
- Petri dishes containing PDA culture medium
- Sporulating fungal cultures
- Glassine envelopes
- Pipettes
- Petri dishes
- Filter paper
- Spatula
- Tweezers
- Marker pen

## Processing steps

- ***Step 1***
  Take a petri dish containing a sporulated fungal strain and add 2 mL of peptone-sucrose solution.

- ***Step 2***
  Use a sterilized spatula or the same pipette that added the solution to scrape the surface of the fungus, thus detaching conidia and obtaining a conidial suspension.

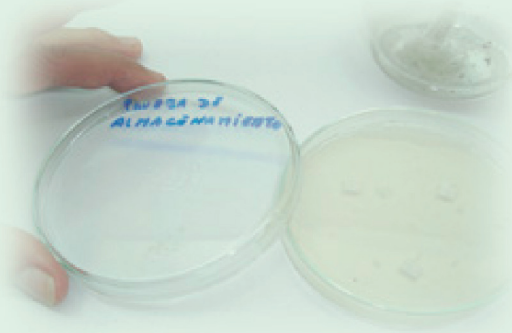
- ***Step 3***
  Use sterilized tweezers to place twenty 0.5-cm squares of sterilized filter paper in a petri dish containing PDA culture medium and impregnate them with the fungus suspended in peptone-sucrose solution.

- ***Step 4***
  Remove the squares from this petri dish, using sterilized tweezers, and place them on filter paper lining the bottom of another, sterilized petri dish. Dry at 24 °C for 7 days.

- ***Step 5***
  After drying, place the squares in sterilized glassine envelopes (**Photo I–34**) for storage. Write on the envelopes information on the fungus such as storage date and strain name (**Photo I–35**). Store at -20 °C.

## G. Proof of storage

This proof is clear, practical, and reliable for guaranteeing that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.



### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with the fungal suspension and dried for 7 days at 24 °C.

- ***Step 2***
  Culture the square on PDA medium. After 5 days, the pathogen should be growing on the medium.

- ***Step 3***
  Examine the fungal growth under the stereoscope. If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

# H. Recovering stored fungus

## *1. Conserved by lyophilization*

### Materials (Photo I–36)

- Peptone-sucrose solution at 20%, prepared as described in ***I.F.1, Step 5***
- Petri dishes containing PDA culture medium
- Sterilized papernapkin
- Bulb pipette
- Pestle
- Tweezers
- Incubator

Photo I–36

Photo I–37

Photo I–38

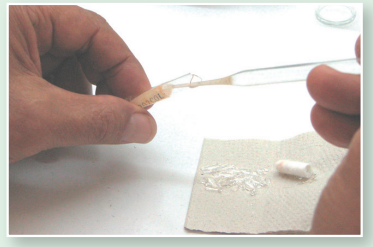


Photo I–39

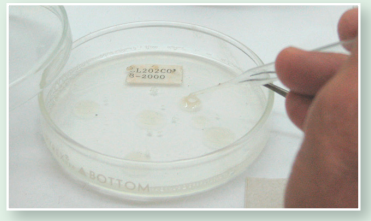


Photo I–40

## Processing steps

- ***Step 1***
  Select a vial containing the lyophilized fungus to be recovered. Use a pestle to break the vial by tapping it at the end opposite to that containing the lyophilized fungus (**Photo I–37**). Use the cloth napkin to cushion the blows and contain the breaking glass. Remove the cotton wool plug from inside the vial, using tweezers (**Photo I–38**).

- ***Step 2***
  Use a pipette to deposit, in the open half of the vial, 4 drops of peptone-sucrose solution. The lyophilized fungal cells will then become suspended in the solution and can then be removed from the vial (**Photo I–39**).

- ***Step 3***
  Transfer the fungal suspension from the vial to a petri dish containing PDA culture medium to reinitiate fungal growth (**Photo I–40**).

## 2. *Conserved as fungal growth on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***I.F.2, Step 7***) containing squares of filter paper carrying the fungal strain to be recovered. Then use sterilized tweezers to select a square and transfer it to a petri dish containing PDA culture medium (**Photo I–41**).

- ***Step 2***
  Use a pipette to deposit 1 or 2 drops of peptone-sucrose solution on the selected square, thus hydrating the fungus. Then use the pipette tip to spread the isolate over the entire surface of the medium (**Photo I–42**). This will increase the area over which the fungus will grow.

- ***Step 3***
  Incubate the petri dish at 20 °C. The fungus will reactivate and, after 12 days, will be ready for use in later procedures.

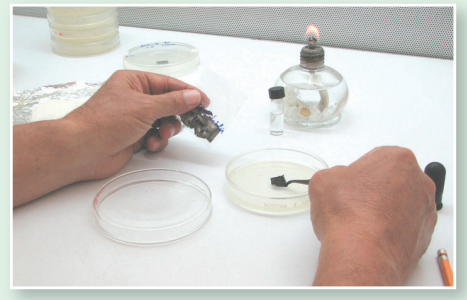


Photo I–41

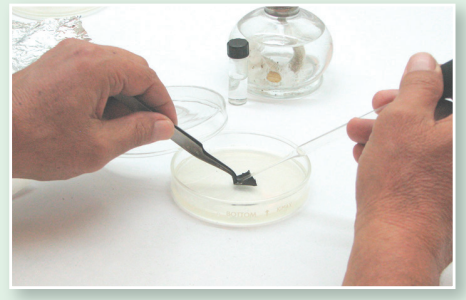


Photo I–42

## *3. Conserved as a suspension on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***I.F.3, Step 5***) containing squares of filter paper carrying the fungal isolate to be recovered. Remove 1 or 2 squares and transfer them into petri dishes containing PDA culture medium.

- ***Step 2***
  Incubate the petri dishes at 20 °C. After 2 or 3 days, fungal growth should be seen. Ten days after incubation, the fungus should be ready for increasing.

# Practical Guide 2

***Xanthomonas axonopodis* pv. *phaseoli***

(Common bacterial blight)

# Managing the bacterium in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
*Since 1967 / Science to cultivate change*

## Contents

# Considerations

Photo II–1

Common bacterial blight is caused by *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Dye and by *Xanthomonas phaseoli* var. *fuscans* (Burkholder) Starr et Burkholder, also called *Xap* and *Xpf*, respectively. Optimal temperatures for their development range between 28 and 30 °C.

Although these two bacteria induce identical symptoms on leaves, stems, and pods of the common bean, their growth on culture media presents different characteristics: *Xpf* produces a coffee-colored pigment after 24 h on YCDA medium, whereas *Xap* does not produce such pigments.

Symptoms of common bacterial blight are as follows:

- On **leaves**, initial symptoms are watery spots that usually appear on the lower surface. These spots increase in size to form large lesions (**Photo II–1**).

- On **pods**, symptoms are small wet spots that, as they grow, join up and cover large areas of pod. The pods become deformed and the seeds inside infected.

Infected tissues become flaccid and are edged by yellow borders that, after a certain time, become coffee colored and eventually cover large areas of the leaf or pod. The disease is transmitted by contaminated seeds.

# Procedures

## A. Collecting and shipping samples

To isolate the bacteria, first collect either bean leaves or pods presenting typical symptoms of the disease.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying plant materials
- Marker pen and fieldbook

- ***Paper towels.*** Wrap collected samples of diseased bean pods, leaves, or stems in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples of diseased tissues, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would favor the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach the labels to their corresponding samples.

## *Recommendations*

- Indicate on the label if collection was in a farmer's field or on an experimental station.
- If there are not enough labels for all the samples, code the bags and record the full information in a fieldbook.
- Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once in the laboratory, and if they are not to be immediately processed, leave them out to finish drying on paper towels spread on a bench.

## Processing steps

- ***Step 1***
  Take samples of leaves or pods from plants presenting common bacterial blight symptoms (***see II.A.***).

- ***Step 2***
  Wrap each sample in a paper towel, place it in a paper bag or envelope, and firmly attach a label carrying the corresponding information (***see II.A.***).

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the bacteria will be isolated.

## B. Preparing YCDA culture medium

The letters in the abbreviation "YCDA" correspond to components of the medium used for isolating the bacteria: yeast, calcium carbonate, dextrose, and agar.

### Materials

- 10 g of yeast extract
- 10 g of dextrose
- 15 g of agar
- 2 g of calcium carbonate ($CaCO_3$)
- 1000 mL of distilled water
- Two 1000-mL erlenmeyer flasks
- Petri dishes
- Large receptacle (e.g., beaker)
- Autoclave
- Balance

### Preparation

- Weigh the ingredients, place them in a large receptacle such as a beaker, and add distilled water. Pour 500 mL of the resulting solution (YCDA culture medium) into each erlenmeyer flasks.
- Autoclave the flasks containing the medium for 40 min at 121 °C and a pressure of 20 psi until sterilized.
- Leave the sterilized medium until it is cool enough to pour into petri dishes at 20 mL per dish.

## C. Isolating *Xap* and *Xpf* bacteria

### Materials (Photo II–2)

- Small petri dishes or clean tubes, either plastic or glass, for macerating samples
- Petri dishes containing YCDA culture medium
- Laminar-flow chamber
- Sterilized distilled water
- Samples of diseased plant material
- 2.5% sodium hypochlorite
- Pasteur pipette
- Scissors
- Cotton wool impregnated with 70% alcohol
- Inoculation needle
- Incubator
- Glass rod or other instrument for macerating

***Note:*** All procedures must be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

### Processing steps

- ***Step 1***
  Clean a pair of scissors with cotton wool already impregnated with 70% alcohol. Then cut the diseased tissue into several small pieces (**Photo II–3**).

Photo II–2

Photo II–3

Photo II–4

Photo II–5

Photo II–6

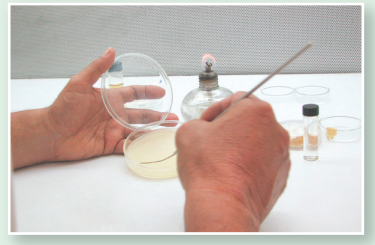

Photo II–7

- ***Step 2***
  Disinfect the pieces by immersing them for 3 min in 2.5% sodium hypochlorite. Then remove excess sodium hypochlorite by washing the sample with sterilized distilled water, using a sterilized Pasteur pipette (**Photo II–4**).

- ***Step 3***
  Macerate the sample in a small petri dish (**Photo II–5**) or tube, using a sterilized glass rod. Then add 10 drops of sterilized distilled water to obtain a suspension of the macerated tissue.

- ***Step 4***
  Use an inoculation needle to take up the suspension of macerated tissue (**Photo II–6**) and streak it across the YCDA medium already placed in a petri dish (**Photo II–7**). Repeat the procedure for other petri dishes.

- ***Step 5***
  Incubate all the dishes at 28 °C for 36 h. By this time, the bacteria will have grown over the medium.

- ***Step 6***
  Use a stereoscope to select a single bacterial colony that is growing separately from the rest of the resulting culture (as shown by arrows in **Photo II–8**). Transfer it to another petri dish containing YCDA medium and streak that new surface in the patterns illustrated in **Figure II–1**. Proceed likewise with colonies obtained from other cultures of the sample.

  Contaminant cultures have visually different morphologies to the targeted bacteria, making it feasible to select and obtain pure colonies for later multiplication.

## Technique for streaking bacteria

To isolate bacteria from a sample of plant tissue or to increase a bacterial culture, follow the pattern of streaks shown in **Figure II–1**, using a sterilized inoculation needle. This method leads to the development of isolated colonies. It also facilitates differentiation between contaminant bacteria and the targeted bacteria.

**Photo II–8**

**Figure II–1**

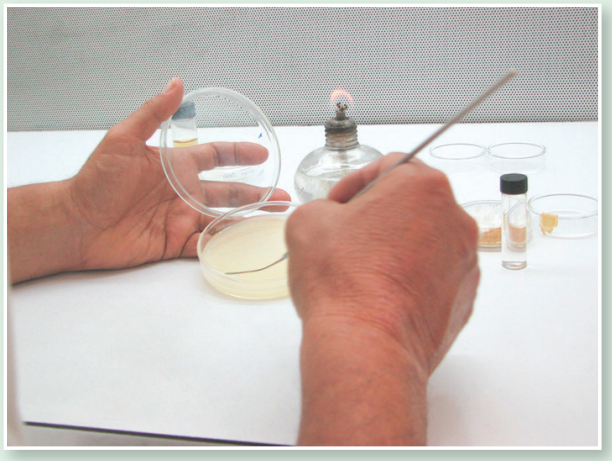

Photo II–9

## D. Increasing *Xap* and *Xpf*

*Xanthomonas* bacteria multiply from colonies that are 24 to 48 h old. Transfer a colony by streaking the selected colony material on YCDA culture medium, using a sterilized inoculation needle (**Photo II–9**). Then incubate the streaked bacteria at 28 °C for 24 to 48 h. Make as many multiplications as needed for the number of plants to be inoculated, whether in the field or greenhouse. Quantities are suggested below (***II.E.3.***).

## E. Inoculating plants

### Materials

- Petri dishes containing bacterial cultures on YCDA culture medium
- Healthy bean leaves
- Cork or similar soft material
- 17-day-old bean plantlets
- Plastic foam
- Distilled water
- Sterilized petri dishes
- Bent-glass streaking rod
- Spectrophotometer
- Large receptacle
- Motorized spray pump
- 5 needles

## 1. Producing inoculum

This procedure requires several cultures of 48-h-old bacteria grown in petri dishes.

### Processing steps

- ***Step 1***
  Add 3 to 5 mL of distilled water to each petri dish containing the bacterial growth obtained in the inoculum preparation step (**Photo II–10**). Then scrape the surface of each culture to detach bacterial colonies from the medium, using a sterilized, bent-glass, streaking rod (**Photo II–11**).

- ***Step 2***
  Collect into one sterilized receptacle all the suspensions from the petri dishes of the previous step. Then use a spectrophotometer to measure the concentration of bacteria in the resulting suspension. Adjust the reading of the suspension to 0.5 units of absorbance to a wavelength of 620 nm. This is equivalent to a concentration of about 5 x $10^8$ colony-forming units per milliliter (cfu/mL).

- ***Step 3***
  Dilute the suspension described in *Step 2* above by 10 times to obtain a final concentration of 5 x $10^7$ cfu/mL. This is the concentration recommended for conducting artificial inoculations. For example, dilute 100 mL of suspension in 900 mL of water.

**Photo II–10**

**Photo II–11**

Photo II–12

Photo II–13

Photo II–14

## 2. Inoculum for use in the greenhouse

### Processing steps

- ***Step 1***
  Prepare materials as follows:
  - Insert the eye end of 5 needles into a cork or stopper of similar soft material so that 1.5 cm of each sharp end protrudes. The cork is now ready for perforating leaf tissue for inoculation.
  - Line a petri dish with an equal-sized circle of plastic foam (**Photo II–12**).
  - Select 17-day-old plants from the greenhouse. They should show a developed primary trifoliate leaf that has expanded by about 70%.

- ***Step 2***
  To inoculate, do the following:
  - Pour a volume of inoculum suspension (see *Step 3* above) on the foam until it is entirely impregnated. Ensure that it does not spill from the edges.
  - Place the selected trifoliate leaf (or leaflet) on the foam.
  - Perforate the leaf, using the needles in the cork. Press the needles down and up to the bottom of the dish so that, on removing them, the bacteria will come into contact with the wounds in the leaf and begin infection (**Photo II–13**).
  - Leave the inoculated plants in the greenhouse at room temperature, with daily temperatures ranging between 25 and 30 °C, until evaluation.

- ***Step 3***
  Evaluate the plants on day 15 after inoculation (**Photo II–14**) using the **CIAT standard scale**, with scores ranging from 1 to 9, where:

  1 to 3 indicate a resistant plant
  4 to 6 indicate a plant with an intermediate reaction
  7 to 9 indicate a susceptible plant

## 3. Inoculum for use in the field

About 50 petri dishes containing 48-h-old bacteria grown on petri dishes are needed for every hectare of crop to be inoculated. Inoculum for spraying should be prepared as follows:

- ***Step 1***
  Obtain a suspension of bacteria from petri dishes growth as described above (***II.E.1, Step 3***) and adjust the concentration to a final volume of 12 L for a motorized spray pump.

- ***Step 2***
  Apply 10 to 12 pumps per hectare, according to the crop's age. Inoculation is recommended at 25 days after planting and again one week afterwards, during flowering.

# F. Conserving the bacteria for storage

The bacteria *Xap* and *Xpf* are conserved either by lyophilization or on pieces of filter paper impregnated with bacteria suspended in peptone-sucrose solution and kept in glassine envelopes.

## *1. Lyophilization*

This is the most reliable method for conserving microorganisms in storage.

### Materials

- Manifold lyophilizer with two types of support (rack and tree) and 48 ports for vials. Other models can also be used according to manufacturer's specifications.
- 0.5-mL neutral-glass vials for lyophilizing, and rack
- Bacterial cultures growing on YCDA medium
- Long Pasteur pipettes
- Peptone at 10%
- Scissors
- Sucrose or dextrose at 20%
- Filter paper
- Tweezers or inoculation needle
- Cotton wool
- Large receptacle
- Marker pen or pencil and notebook
- Distilled water
- Propane gas torch
- Autoclave

– Freezer
– Bunsen burner

***Note:*** The bacterial strains to be lyophilized must be grown for 48 h at 28 °C before this procedure takes place (**see *II.D.***).

## Processing steps

- ***Step 1***
  Write in either small lettering or code on 1- to 2 cm squares of filter paper to identify the isolates to be lyophilized. Include the bacterium's name (either *Xap* or *Xpf*), the isolate's number (i.e., the code the laboratory uses), and storage date.

  If you write in code, write the full information in a notebook.

- ***Step 2***
  Use sterilized scissors to cut out the pieces of filter paper identifying each bacterium. Introduce each piece to the bottom of a vial.

- ***Step 3***
  Then prepare a cotton wool plug to stopper each vial so that it plugs the top two thirds, as follows:
  – Tease out a piece of cotton wool until it measures about 5 x 3 cm and roll it around a tweezers arm or an inoculation needle, pressing between fingers and thumb until the plug is formed (**Photos II–15**, **II–16**, and **II–17**). Leave a piece of unrolled cotton wool on the end opposite the plug.

**Photo II–15**

**Photo II–16**

**Photo II–17**

- Introduce the plug into the vial and gently withdraw the tweezers arm or needle with one hand while holding the plug in the vial with the other.
- Prepare additional vials with their respective plugs to have spares on hand as needed. If a plug disintegrates on handling, it can therefore be replaced by one from the additional vials.

- ***Step 4***
  Autoclave all the vials, with their respective plugs, for 40 min until sterilized.

- ***Step 5***
  **Preparing peptone and sucrose solutions.**
  Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a solution of either sucrose or dextrose at 20% by dissolving 20 g of the sugar in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix the two solutions in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose (or peptone-dextrose) solution.

- ***Step 6***
  Use a long Pasteur pipette to draw up 2 to 3 mL of the peptone-sucrose solution and deposit them on a bacterial culture. Homogenize the bacterium within the solution by repeatedly drawing up and expelling the mixture, using the same pipette.

- ***Step 7***
  Use the same pipette to draw up 2 to 3 mL of this homogenized suspension and deposit them inside a vial, as follows:
  – In one hand take the Pasteur pipette and, in the other, the vial with its respective plug as prepared in *Step 3* above.
  – Hold the Pasteur pipette between the index finger and thumb, and, using the little finger (or pinky) of the same hand, carefully withdraw the cotton wool plug from the vial by its loose end, keeping the plug firmly against the palm. Immediately deposit the bacterial suspension in the bottom of the vial, which should have the piece of filter paper identifying the bacterium.
  – Stopper the vial by gently introducing the plug back into it.

  If the plug comes into contact with another surface or substance, it becomes contaminated. Discard it therefore and replace it by one of the additional plugs that had been prepared in *Step 3* above for such an eventuality.

- ***Step 8***
  Use flame-sterilized scissors to cut off that part of the plug remaining outside the vial (**Photo II–18**). Then introduce the rest of the plug into the vial, using the point of a scissors arm, until 1 cm is left between the lip of the vial and the plug (**Photo II–19**).

**Photo II–18**

**Photo II–19**

Photo II–20

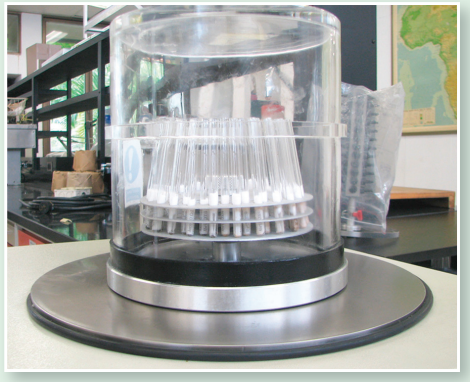

Photo II–21

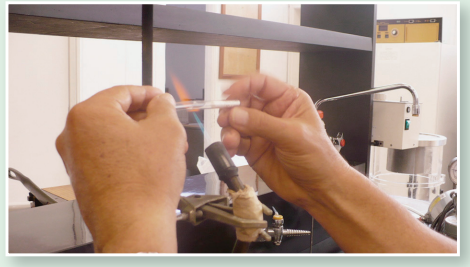

Photo II–22

- ***Step 9***
  Place the vials in a rack. Then push the plug to the bottom of each vial until it touches the piece of filter paper identifying the bacterial strain. To do this, use a long object such as an inoculation needle, constantly flame-sterilizing it to prevent contaminating the plugs (**Photo II–20**). The upper part of the vial, which has no plug, is sealed after lyophilization (see *Step 14* below).

- ***Step 10***
  Once the vials are organized, freeze them at 0 °C for 15 min. When the samples are frozen, lyophilization is initiated.

- ***Step 11***
  Remove the rack from the freezer and place it inside the lyophilizer dome. Switch on the machine (**Photo II–21**). The temperature inside the apparatus will drop to -55 °C at which point a vacuum pump will begin extracting water, drying the samples. This procedure takes 20 to 22 h to complete.

- ***Step 12***
  The next day, switch off the lyophilizer and remove the rack of vials. Prepare a propane gas torch for constricting the vials in the part opposite the sample. That is, make a neck in each vial by softening the glass with the torch's flame and pulling gently on the ends of the vial (**Photos II–22 and II–23**).

The procedure requires considerable care to prevent burns to the hands. This step reduces the space by which air and therefore humidity can reach the sample before sealing is completed.

- ***Step 13***
  Once all the vials are constricted, place them on the ports of the lyophilizer tree, inserting the open end into each port. Thus, the end carrying the sample remains visible (**Photo II–24**). Repeat the drying process in the lyophilizer for about 1 h to eliminate any humidity that may have reached the sample during *Step 12*.

- ***Step 14***
  With the lyophilizer still operating, seal the vials, one by one, by vacuuming and then using the propane gas torch to melt the glass at the neck (**Photo II–25**). Once the vials are sealed, lyophilization is completed.

***Note:*** If the vacuum is lost on sealing a vial, remove this part of the vial and replace it with a new one. Wait for the vacuum to reach the point of sealing before continuing to seal the other vials.

How long a lyophilized sample can last in storage has not been formally established. However, in our laboratory, lyophilized samples have been recovered after 30 years of storage. Lyophilized samples do have a disadvantage: to recover a sample, the entire vial must be broken. Several copies of each lyophilized sample must therefore be made to maintain an adequate collection.

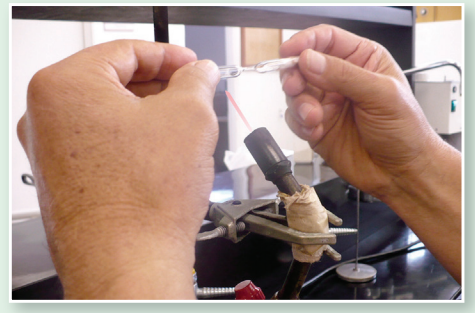

**Photo II–23**

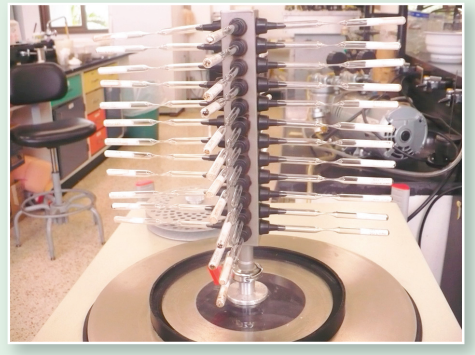

**Photo II–24**

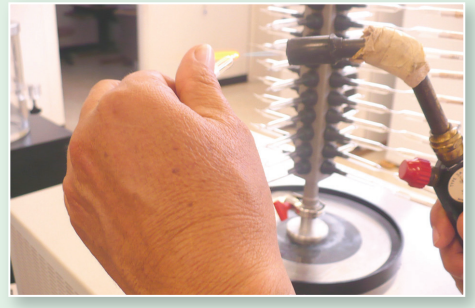

**Photo II–25**

## *2. Conservation as a suspension*

The next most effective storage method after lyophilization is to conserve a pathogen in suspension either peptone-sucrose or peptone-dextrose solution, and deposit the suspension on pieces of filter paper for storage at -20 °C.

### Materials (Photo II–26)

- Peptone-sucrose or peptone-dextrose solution, prepared as described in ***II.F.1., Step 5***
- Petri dishes carrying bacterial cultures
- Tweezers
- Filter paper
- Sterilized petri dishes
- Spatula
- Glassine envelopes
- Pasteur pipettes
- Marker pen or pencil
- Scissors

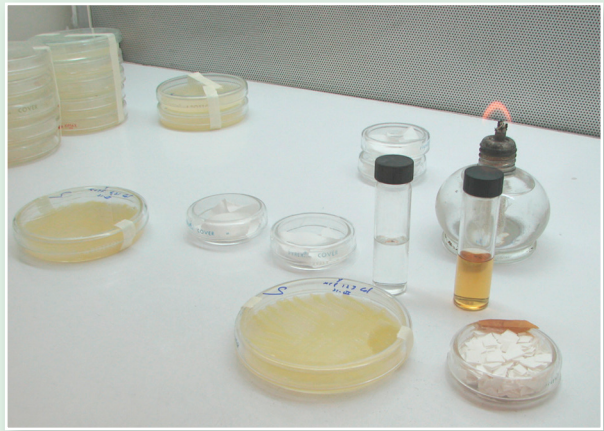

Photo II–26

## Processing steps

- ***Step 1***
  Take a petri dish containing a 48-h-old bacterial culture and add 2 mL of either peptone-sucrose or peptone-dextrose solution (**Photo II–27**).

- ***Step 2***
  Use a spatula (or the same pipette used to add the solution) to scrape the bacterial culture, detaching colonies. Suspend the colonies in the solution just added (**Photo II–28**).

- ***Step 3***
  Within the suspension of bacterial colonies, place 20 or more pieces of sterilized filter paper, previously cut into 0.5-cm squares, and let them become impregnated with the suspension (**Photo II–29**).

- ***Step 4***
  Use sterilized tweezers to remove squares of filter paper from the petri dish and place them on filter paper lining the bottom of another, sterilized petri dish. Dry at 24 °C for 7 days.

- ***Step 5***
  After drying, place the squares of paper in sterilized glassine envelopes for storage (**Photo II–30**). Write on the envelopes information on the bacterium such as name of isolate, place of origin, and storage date (**Photo II–31**). Store at 20 °C.

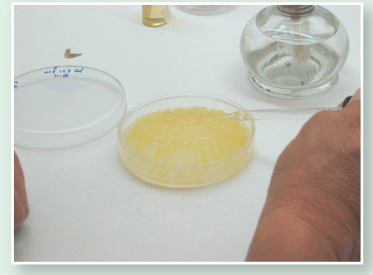

**Photo II–27**

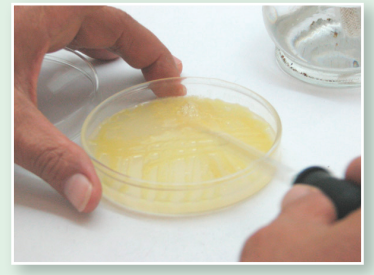

**Photo II–28**

**Photo II–29**

**Photo II–30**

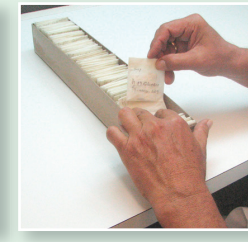

**Photo II–31**

Photo II–32

Photo II–33

Photo II–34

# G. Recovering stored bacteria

## Materials (Photo II–32)

- Peptone-sucrose or peptone-dextrose solution, prepared as described in *II.F.1, Step 5*
- Petri dishes containing YCDA culture medium
- Bulb pipette
- Sterilized inoculation needle
- Sterilized cloth napkin
- Tweezers
- Pestle
- Incubator

## *1. Conserved by lyophilization*

### Processing steps

- ***Step 1***
  Select a vial containing the lyophilized bacterium to be restored. Use a pestle to break the vial by tapping the end opposite that containing the lyophilized bacterium (**Photo II–33**). Use the cloth napkin to cushion the blows and contain the breaking glass. Then remove the cotton wool plug from the vial, using tweezers (**Photo II–34**).

- ***Step 2***
  With a pipette, deposit 4 drops of peptone-sucrose or peptone-dextrose solution on the sample in the vial. The lyophilized bacterial cells will then become suspended in the solution (**Photo II–35**).

- ***Step 3***
  Remove the suspension of cells from the vial and culture it into a petri dish containing YCDA culture medium (**Photo II–36**). Spread the bacteria by streaking (**Figure II–1, p. 2–9**) with a sterilized inoculation needle and incubate at 28 °C. In 48 h, the bacterium will have grown over the medium.

## 2. Conserved as suspension in solution

A stored bacterium that has been conserved through this protocol is best reactivated by depositing either peptone-sucrose or peptone-dextrose solution on the bacterium. The solution will provide nutrients, allowing the bacterium to resume growth.

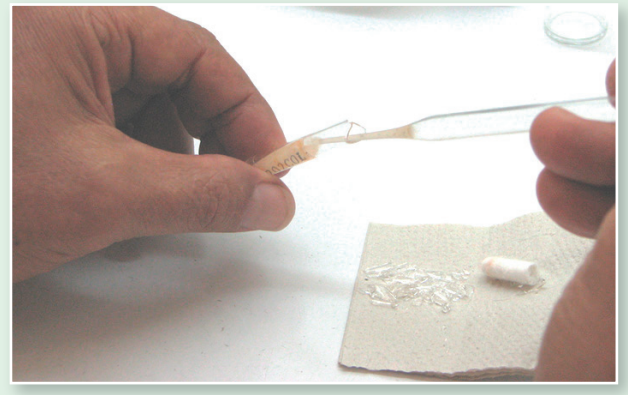

**Photo II–35**

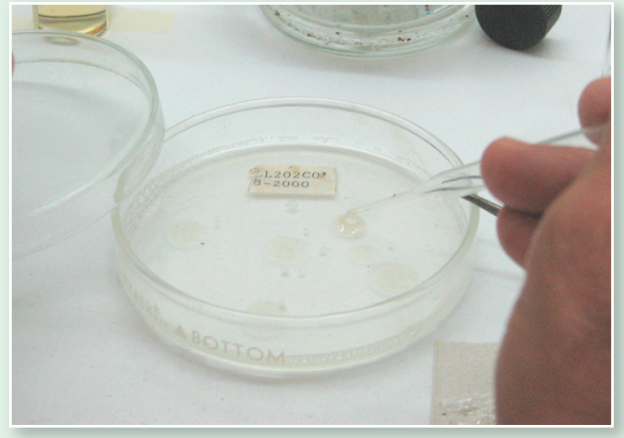


**Photo II–36**

Photo II–37

## Processing steps

- ***Step 1***
  Select a glassine envelope (see **Photo II–31**) containing squares of filter papers carrying the bacterial isolate to be restored. Remove 3 to 5 squares from the envelope and transfer them to a petri dish containing YCDA culture medium (**Photo II–37**).

- ***Step 2***
  Deposit a drop of either peptone-sucrose or peptone-dextrose solution on each square. Then streak the bacterium (**Figure II–1, p. 2–9**)—among the squares—across the medium, using an inoculation needle.

- ***Step 3***
  Incubate the petri dishes at 28 °C. After 2 days, the bacterium should have resumed its growth and be ready for multiplication.

# Practical Guide 3

*Colletotrichum lindemuthianum*

(Anthracnose)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
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## Contents

# Considerations

Photo III–1

The fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara causes bean anthracnose, a disease found throughout the world. Favorable conditions include temperatures ranging between 13 and 25 °C, and high relative humidity.

The disease can be devastating for the small farmer, causing total crop loss not only in beans but also in other susceptible vegetables (**Photo III–1**).

## Symptoms

Symptoms of anthracnose infection may appear on any plant part depending upon time of infection and source of inoculums. Infected seed and crop debris are primary source inoculums for local epidemics. Initial symptoms may, in fact, appear on the cotyledonary leaves as small, dark brown to black lesions. Conidia and hyphae then my be transported by rain or dew to the developing hypocotyls where infection causes minute flesh to rust-colored specks. The specks gradually enlarge lengthwise along, and partially around, the hypocotyls and young stem, forming a sunken lesion. Pod infections appear as flesh to rust-colored lesions which develop into sunken cankers (1–10 mm in diameter) delimited by slightly raised black rings surrounded by a reddish brown border.

# Procedures

## A. Collecting and shipping samples

To isolate the fungus *C. lindemuthianum*, first collect bean stems, leaves, or pods presenting typical symptoms of the disease. On leaves, the lesions follow the veins and are highly visible on the lower surface (**Photo III–2**).

**Photo III–2**

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying the materials
- Marker pen and fieldbook

- ***Paper towels.*** Wrap collected samples of diseased tissue (from pods, leaves, or stems) in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples of diseased tissue, already wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying the samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach the labels to their corresponding samples.

## *Recommendations*

- Indicate on the label if collection was in a farmer's field or on an experiment station.
- If there are not enough labels for all the samples, code the bags and record the full information in a fieldbook.
- Do not collect wet plant materials. If this is unavoidable before shipping, dry them with paper towels. Once in the laboratory, and if they are not to be immediately processed, leave them out to finish drying on paper towels spread on a bench.

### Processing steps

- ***Step 1***
  Take samples of leaves, pods, or stems from plants presenting anthracnose.
- ***Step 2***
  Wrap each sample in a paper towel, put it into a paper bag or envelope, and firmly attach the corresponding label.
- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons explained above.

## B. Preparing PDA culture medium

The letters in the abbreviation "PDA" correspond to components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- PDA powder (39 g)
- 1 L sterilized distilled water
- Two 1000-mL erlenmeyer flasks
- 50 petri dishes
- Large receptacle (e.g., beaker)
- Autoclave
- Balance

Photo III–3

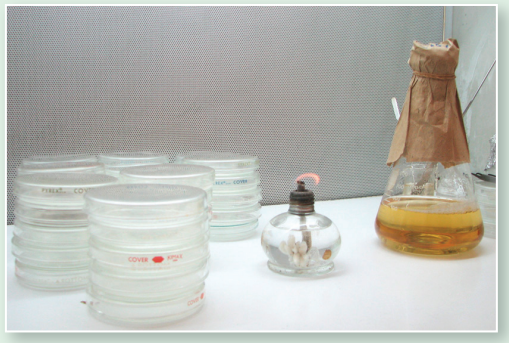


Photo III–4

Photo III–5

### Preparation

- Weigh the ingredients (**Photo III–3**), place them in a large receptacle such as a beaker, and add distilled water. Pour the resulting solution (PDA culture medium) into two erlenmeyer flasks at 500 mL each.
- Autoclave the flasks containing the medium for 40 min at 121 °C and a pressure of 20 lb until sterilized.
- Leave the sterilized medium (**Photo III–4**) until cool enough to pour into petri dishes at 20 mL per dish (**Photo III–5**).

## C. Isolating *C. lindemuthianum*

### Materials

The materials for the two procedures using PDA culture medium include:

- Samples of diseased tissues
- Petri dishes containing PDA culture medium
- Laminar-flow chamber
- Bunsen burner
- Incubator
- Tweezers
- Scissors
- 60- and 100-mm petri dishes
- Paper towels
- Pipette
- Stereoscope
- Sterilized distilled water

– 2.5% sodium hypochlorite
– Bent-glass streaking rod
– Needle

## *1. On PDA culture medium*

***Note:*** All procedures for this technique should be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

### Processing steps

- ***Step 1***
  Use sterilized scissors to cut a sample of diseased tissue into several small pieces. The sample should show typical and well-developed lesions (**Photo III–6**).

- ***Step 2***
  Disinfect the sample pieces for 3 min in a 60-mm petri dish containing 2.5% sodium hypochlorite. Then take up another petri dish and rinse the disinfected pieces several times with sterilized distilled water, using a sterilized pipette (**Photo III–7**).

- ***Step 3***
  Place the disinfected and washed pieces on sterilized paper towels to dry for 10 min (**Photo III–8**).

**Photo III–6**

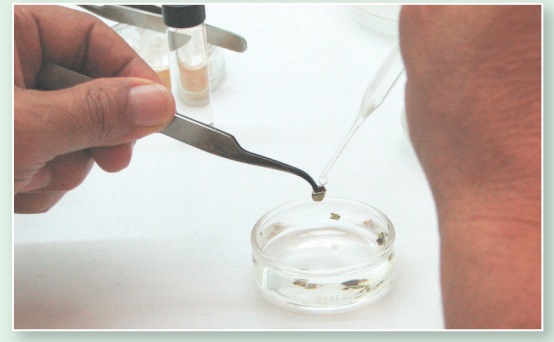


**Photo III–7**

**Photo III–8**

Photo III–9

- ***Step 4***
  Once the pieces are dry, use sterilized tweezers to select 3 pieces and culture them into a petri dish containing PDA medium (**Photo III–9**). Continue with the other pieces until all are cultured.

- ***Step 5***
  Incubate the petri dishes at 20 °C for 10 days.

If a cultured sample piece contains contaminant microorganisms, the fungus sometimes will still to grow. In this case the fungus can then be purified by taking portions from the fungi, free of contaminants, and transferring them to new petri dishes containing PDA culture medium. Repeat this process until a pure fungal growth is obtained.

After 10 days of incubation, the fungus will have produced acervuli. These reproductive structures shelter a large quantity of conidia, some of which can be used to produce monosporic isolates, as described in the next section.

## 2. As a monosporic culture

Because a sample of diseased tissues may carry a mixture of strains, the genetic purity of a *C. lindemuthianum* isolate is assured only when that isolate is generated from a single conidium.

### From growth on PDA culture medium

The methodology applied for this procedure is as follows:

- ***Step 1***
  Use a stereoscope to examine the diseased tissue cultured on PDA medium (from *Step 4* above). Identify well-developed acervuli, which should be salmon colored and carrying conidiophores and conidia. These are easily released when water comes into contact with the acervuli.

- ***Step 2***
  Collect, by touching with a flame-sterilized needle, a conidial mass from a selected acervulus. Ensure that several conidia will adhere to the needle point.

- ***Step 3***
  Remove the conidia from the needle by washing them with 4 to 6 drops of sterilized distilled water from a pipette held over PDA culture medium in a petri dish. Spread the conidia-containing drops across the medium's surface, using a bent-glass streaking rod to assure enough isolation between conidia

  Ensure that the streaking rod is flame-sterilized before each use to prevent contaminating the medium.

- ***Step 4***
  Incubate the petri dishes at 20 °C for 24 h, by which time the conidia should begin germinating.

- ***Step 5***
  Examine the germinated conidia under the stereoscope, select one, and remove it with a flame-sterilized needle, transferring it to a petri dish containing PDA culture medium.

- ***Step 6***
  Incubate the petri dish containing a single grminated conidium at 20 °C. The conidium should grow on this medium so that, after 12 days, the fungus completely covers the medium.

## From growth on pods or stems in a humid chamber

After collecting samples of diseased pods or stems, isolate fungal strains by inducing sporulation. Conduct this procedure inside a humid chamber.

### Materials

To set up a small humid chamber, use the following materials:

- Petri dishes
- Sterilized distilled water
- Filter paper
- V-shaped glass rod
- Sample of diseased stem or pod

### Processing steps

- ***Step 1***
  Place filter paper in a sterilized petri dish and wet it with distilled water.

- ***Step 2***
  Put a V-shaped glass rod (**Photo III–10**) on the wetted paper and place, on the rod, a piece of diseased pod or stem presenting acervuli. Ensure that the diseased tissue does not come into contact with the wet paper.

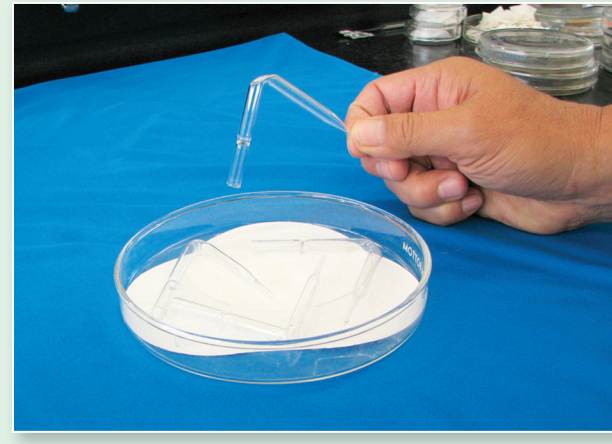


**Photo III–10**

- ***Step 3***
  Leave the petri dish for 2 days in a dark place at room temperature to maintain the filter paper humidity.

- ***Step 4***
  The piece of pod or stem should produce acervuli within this humid environment.

- ***Step 5***
  Obtain a monosporic isolate as described above.

## D. Increasing *C. lindemuthianum*

### *1. On PDA culture medium*

A monosporic isolate enables multiplication of a given strain (see *III.C.2.* above). Add sterilized distilled water to a petri dish containing a monosporic strain that has sporulated effectively. Use a spatula to disrupt the acervuli and thus form a conidial suspension. Then, with a pipette, transfer several drops of the suspension to fresh petri dishes containing PDA culture medium. Streak the drops with a flame-sterilized rod to encourage the fungus to grow over the entire medium (**Photo III–11**).

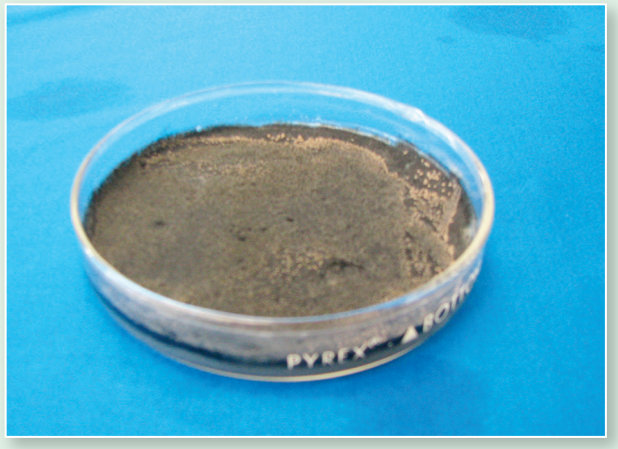


**Photo III–11**

## 2. On bean leaves

Although *C. lindemuthianum* isolates will grow on PDA culture medium, some do not sporulate sufficiently. In some cases this limitation can be improved by growing the fungus on sterilized bean leaves. The laboratory should register which isolates require this supplementary procedure and which grow on simple PDA media.

### Preparing leaves

Select only those bean leaves that are free of fungicides, are mature at 30 days old, and are medium sized. Note that young leaves disintegrate when autoclaved, and very large leaves will not fit into petri dishes. Place the selected leaves in large petri dishes with the abaxial surface uppermost and spray with a little distilled water. Wrap the petri dishes, first in aluminum foil and then in wrapping paper, and autoclave them.

### Materials

- Fungal isolates with 10 days of growth on PDA culture medium
- Healthy, sterilized bean leaves free of fungicides
- Petri dishes containing PDA culture medium
- Sterilized distilled water
- Bulb pipette
- Tweezers
- Spatula

Photo III–12

Photo III–13

Photo III–14

- Bunsen burner
- Incubator
- Glass inoculation needle

## Processing steps

- ***Step 1***
  Use sterilized tweezers to select a sterilized bean leaf from the large petri dish. Place it in a medium-sized petri dish containing PDA culture medium (**Photo III–12**).

- ***Step 2***
  Use a bulb pipette to add sterilized distilled water to a monosporic strain growing on culture medium (**Photo III–13**). With a spatula, scrape the surface of the isolate to obtain a suspension of *C. lindemuthianum*.

- ***Step 3***
  Draw up several drops of the fungal suspension and deposit them on the bean leaves already placed on medium (**Photo III–14**). Spread the suspension across the entire leaf area, using a flame-sterilized glass inoculation needle.

- ***Step 4***
  Incubate the petri dishes at 20 °C for 12 days, by which time, the fungus will have grown and produced abundant acervuli.

  **Photo III–15** shows a *C. lindemuthianum* isolate growing on bean leaves. The salmon-colored sporulation can be seen on the leaves.

**Photo III–15**

## 3. On bean pods

Because plant inoculation in the field requires a large volume of inoculum suspension, *C. lindemuthianum* fungus is grown on bean pods.

***Note:*** This method of multiplication requires fungal isolates growing on PDA culture medium and already sporulating.

### Materials

- Sporulated fungal isolate growing on PDA culture medium
- Healthy bean pods
- Sharp knife
- Sterilized distilled water
- Marker pen

Photo III–16

Photo III–17

- 250-mL erlenmeyer flasks
- Sterilized cotton wool
- Aluminum foil
- Wrapping paper
- Rubber bands
- Pasteur pipette
- Skilful fingers
- Running water
- Tweezers
- Spatula
- Laminar-flow chamber
- Autoclave
- Incubator

## Processing steps

- ***Step 1***
  Wash bean pods under running water to eliminate residues of agrochemical products and other possible contaminants (**Photo III–16**).

- ***Step 2***
  Cut the bean pods into pieces of no more than 1 cm long (**Photo III-17**), using a sharp knife.

- ***Step 3***
  Fill erlenmeyer flasks to the neck with the bean-pod pieces (**Photo III–18**). Stopper the flask with a cotton wool plug (**Photo III–19**), and cover the flask mouth and plug with aluminum foil and then wrapping paper. Adjust the entire covering with a rubber band (**Photo III–20**).

  The cotton wool plug is a key piece in this procedure because it maintains the sterility of the flasks' contents. Take care to assemble the plug well, and ensure that it rests firmly against the flask neck so that it will not disintegrate on handling.

- ***Step 4***
  Autoclave the flasks containing the bean-pod pieces two times until sterilized.

- ***Step 5***
  Wait until the flasks are cool and remove the water released from the pod pieces during sterilization (**Photo III–21**). The entire process, from *Steps 1* to *5*, should be carried out in a laminar-flow chamber.

**Photo III–18**

**Photo III–19**

**Photo III–20**

**Photo III–21**

Photo III–22

Photo III–23

- ***Step 6***
  Add sterilized distilled water to the petri dishes containing the sporulated isolate. Scrape, using a spatula, the acervuli from the culture's surface to make a conidial suspension (**Photo III–22**).

- ***Step 7***
  Add the suspension to the sterilized bean-pod pieces, as follows:

  – With a Pasteur pipette in your right hand, fill it with about 2 mL of conidial suspension.
  – Without letting go of the pipette, use your left hand to remove the papers covering the mouth of the erlenmeyer flask and the cotton wool plug. Hold the seals with your fingers of the left hand.
  – Deposit the suspension in the pipette into the flask, spreading it over the bean-pod pieces (**Photo III–23**).
  – Stopper the flask again with the cotton wool plug.

  Good results for this step depend on the cotton wool plug being firmly assembled, as mentioned above in *Step 3*, and not spoiling it when removing it from the flask. If the plug does come apart on being removed and no spare sterilized plug is available, avoid touching it so not to contaminate it. Use tweezers to recover the plug and, as

skillfully as possible, introduce it again into the flask neck to plug it. Make an effort to adjust the plug as much as possible.

Then seal the erlenmeyer flask with the foil-and-paper cap, and the rubber band. Write the isolate's number on the flask.

- ***Step 8***
  Gently strike each flask against the hand so that the conidial suspension covers all bean-pod pieces. Do not allow the suspension to touch the cotton wool plug (**Photo III–24**), as this will reduce the number of propagules on the increasing substrate (i.e., the bean-pod pieces).

- ***Step 9***
  Incubate all the flasks at 20 °C for 9 days, by which time the fungus will have colonized and sporulated on the bean-pod pieces and can be used to prepare inoculum for use in the field.

**Photo III–24**

# E. Inoculating plants

## *1. Inoculum for use in the greenhouse*

### Processing steps


Photo III–25

Photo III–26

- ***Step 1***
  Twelve days before inoculation, multiply the fungus according to the number of plants to be inoculated.

  A petri dish carrying abundant sporulation would produce about 300 mL of inoculum, which would be enough for 3 trays. Calculation, however, depends on the isolate's characteristics, as not all isolates sporulate the same way. Calculate the number of petri dishes containing the fungus grown on PDA medium needed to make the required amount of inoculum. Then start the fungal increase.

- ***Step 2***
  Add sterilized distilled water to petri dishes containing fungal growths. Scrape each culture's surface with a sterilized spatula to detach and suspend the conidia in the water (**Photo III–25**). Filter the conidial suspension through sterilized gauze to separate particles such as agar residues and mycelium. Collect the filtrate containing the conidia in a sterilized medium-sized beaker or large sterilized petri dish (**Photo III–26**).

The filtration will facilitate the conidial count, and also prevent the nozzle of the DeVilbiss nebulizer from blocking. The nebulizer is used, together with a compressor, to apply inoculum to the plants. An airbrush or liquid atomizer can be used instead of a DeVilbiss.

- ***Step 3***

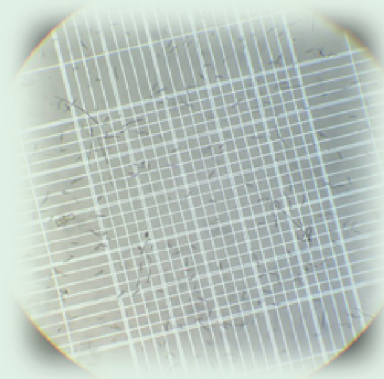
  Use a microscope to count the number of conidia found in the central grid of a hemocytometer, itself comprising one central and four outer squares. Multiply the number of conidia counted by 50,000. The concentration of inoculum should be, in this case, $1.2 \times 10^6$ conidia per milliliter. Given the final volume of inoculum to be sprayed, and given the concentration of conidia in the original inoculum, use the following formula to find the volume of the original inoculum needed for spraying:

  $$V_1 \times C_1 = V_2 \times C_2$$

  For example, if 150 conidia are counted in the hemocytometer, then:

  $$150 \times 50{,}000 = 7{,}500{,}000$$
  $$= 7.5 \times 10^6 \text{ (conidia in 1 mL)}$$

  This is the concentration of original inoculum (i.e., the filtrate in *Step 2*). If 250 mL of inoculum with a concentration of $1.2 \times 10^6$ conidia per milliliter are needed then, by substituting these data in the previous formula, the value of the volume of the original (also called *initial*) inoculum needed (i.e., $V_1$) can be found, that is:



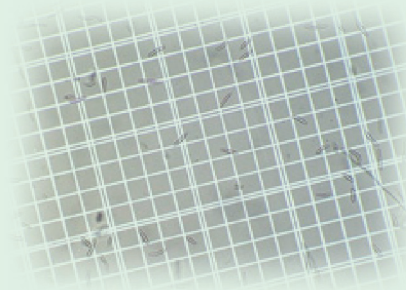


Photo III–27

Photo III–28

Photo III–29

$$V_1 \times 7.5 \times 10^6 = 250 \text{ mL} \times 1.2 \times 10^6$$

Thus,

$$V_1 = \frac{250 \text{ mL} \times 1.2 \times 10^6}{7.5 \times 10^6} = 40 \text{ mL}$$

To obtain the final concentration desired, take the 40 mL of initial inoculum and complete it to 250 mL with sterilized distilled water.

- ***Step 4***
  Once having the inoculum with the required concentration of conidia per milliliter, pour a volume of the suspension into a 250-mL erlenmeyer flask. Connect the flask to a DeVilbiss nebulizer (or airbrush) and this to a compressor (**Photo III–27**). Spray 8 day-old plantlets, thus inoculating them (**Photo III–28**).

- ***Step 5***
  Place the inoculated plants in a humid chamber set at a relative humidity of more than 90% (Photo III–29) and leave until evaluation 8 days after inoculation. To evaluate, use the **CIAT standard scale**, with scores ranging from 1 to 9, where:

  1 to 3 indicate a resistant plant
  4 to 6 indicate a plant with an intermediate reaction
  7 to 9 indicate a susceptible plant

## 2. *Inoculum for use in the field*

### Materials

The volume of the inoculum suspension for inoculations in the field is much higher than the one used for the greenhouse. To prepare sufficient inoculum for spraying 1 hectare of bean crop in the field, the following materials are needed:

- 16 kg of bean-pod pieces carrying abundantly sporulating *C. lindemuthianum* fungus
- Destilled water
- Industrial blender
- Large receptacle
- Medium-sized funnel
- Hemocytometer
- Spatula
- Motorized back sprayer

### Processing steps

- ***Step 1***
  Pour destilled water into the bowl of an industrial blender. Use a spatula to add bean-pod pieces infested with the pathogen and which had been conserved in erlenmeyer flasks. Adjust the amount of water if is required to facilitate the homogenization process.


Photo III–30

- ***Step 2***
  Blend the contents (i.e., the infested bean-pod pieces + water) until a homogeneous product is obtained. Pour it, using a funnel, into an easily handled receptacle. (**Photo III–30**).

- ***Step 3***
  Quantify the conidial concentration in the blended product by using a hemocytometer. Then calculate the volume of the final solution of inoculum according to the area of crop to be inoculated. Adjust the final concentration of inoculum with destilled water.

- ***Step 4***
  Always carry out inoculations with this pathogen in the early evening, when temperatures are dropping and relative humidity is increasing. Use a motorized back sprayer to inoculate the crop.

## F. Conserving the fungus for storage

The fungus *C. lindemuthianum* can be conserved by lyophilization, on pieces of filter paper kept in glassine envelopes, or on pieces of filter paper impregnated with the fungus suspended in peptone-sucrose solution and kept in glassine envelopes.

## *1. Lyophilization*

### Materials (Photo III–31)

- Tree manifold lyophilizer with two types of support (rack and tree) and 48 ports for vials
- 0.5-mL neutral-glass vials for lyophilization, and rack
- Growths of fungus on PDA culture medium
- Receptacle
- Marker pen and notebook
- Distilled water
- Long Pasteur pipettes
- Filter paper
- Scissors
- Tweezers or inoculation needle
- Autoclave
- Bunsen burner
- Peptone at 10%
- Freezer
- Sucrose (or dextrose) at 20%
- Propane gas torch
- Cotton wool

***Note:*** The fungal isolates should be prepared 12 days before lyophilization is carried out (**see *III.D.* above**).

Photo III–31

Photo III–32

Photo III–33

Photo III–34

## Processing steps

- ***Step 1***
  Write in either small lettering or code on 1- to 2-cm squares of filter paper to identify the strains to be lyophilized. Include the fungus's scientific name, strain number (i.e., the code the laboratory uses), and storage date.

  If you write in code, write the full information in a notebook.

- ***Step 2***
  Use sterilized scissors to cut out the pieces of filter paper identifying the isolates, and introduce each piece to the bottom of a vial.

- ***Step 3***
  Then prepare a cotton wool plug to stopper each vial so that it plugs the upper two thirds, as follows:

  – Tease out a piece of cotton wool until it measures about 5 x 3 cm and roll it around a tweezers arm or inoculation needle, pressing between fingers and thumb until a plug is formed (**Photos III–32**, **III–33**, and **III–34**). Leave a piece of unrolled cotton wool on the end opposite the plug.
  – Introduce the plug into the vial and gently withdraw the tweezers arm or needle with one hand while holding the plug in the vial with the other.

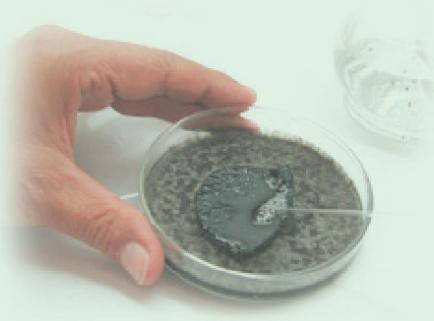
– Prepare additional vials with their respective plugs to have spares on hand as needed. If a plug disintegrates on handling, it can therefore be replaced by one from the additional vials.

- ***Step 4***
  Autoclave all the vials with their respective plugs, together with the pipettes, until sterilized.

- ***Step 5***
  **Preparing peptone and sucrose solutions.**
  Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a solution of either sucrose or dextrose at 20% by dissolving 20 g of the sugar in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix the two solutions in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose (or peptone-dextrose) solution.

- ***Step 6***
  Use a long Pasteur pipette to draw up 2 to 3 mL of the peptone-sucrose solution and deposit them on a fungal culture. Homogenize the fungus within the solution by first scraping the culture with the pipette and then repeatedly drawing up and expelling the mixture, using the same pipette on the same medium. Use only one pipette throughout this procedure (**Photo III–35**).

**Photo III–35**



- ***Step 7***
  Use the same Pasteur pipette to draw up 2 to 3 mL of the homogenized suspension and deposit them inside a vial, as follows:

  – In one hand take the Pasteur pipette and, in the other, the vial with its respective plug as prepared in *Step 3* above.
  – Hold the Pasteur pipette between the index finger and thumb, and use the little finger (or pinky) of the same hand to hold the loose end of the cotton wool plug against the palm. Carefully withdraw the plug from the vial, keeping it firmly against the palm. Then deposit the fungal suspension in the bottom of the vial, which should have the filter paper identifying the strain.
  – Stopper the vial by gently introducing the plug, which had been held between the little finger and palm, back into the vial.

  If the cotton wool plug comes into contact with another surface or some substance, it is contaminated. Discard it therefore and replace it by one of the additional plugs that had been prepared in *Step 3* above for such an eventuality.

- ***Step 8***
  Cut off that part of the plug remaining outside the vial, using flame-sterilized scissors (**Photo III–36**). Then, using a scissors arm, introduce the rest of the plug until 1 cm is left between the lip of the vial and the plug. (**Photo III–37**).

- ***Step 9***
  Place the vials in a rack. Then push the plugs to the bottom of each vial until it touches the piece of filter paper identifying the strain. To do this, use a long object such as an inoculation needle, constantly flame-sterilizing it to prevent contaminating the plugs (**Photo III–38**).

  The upper part of the vial, which has no plug, is sealed after lyophilization (see *Step 14* below).

- ***Step 10***
  Once the vials are organized, freeze them at 0 °C for 15 min. When the samples are frozen, lyophilization is initiated.

**Photo III–36**

**Photo III–37**

**Photo III–38**

Photo III–39

Photo III–40

Photo III–41

Photo III–42

- ***Step 11***
  Remove the rack from the freezer and place it inside the lyophilizer dome. Switch on the machine (**Photo III–39**). The temperature inside the apparatus will drop to -55 °C, at which point a vacuum pump will begin extracting water, drying the samples. This process takes between 20 and 22 h to complete.

  ***Step 12***
  The next day, switch off the lyophilizer and remove the rack of vials. Prepare a propane gas torch for constricting the vials at the end opposite the sample. That is, make a neck in each vial by softening the glass with the torch's flame and pulling gently on the ends of the vial. (**Photos III–40** and **III–41**).

  This procedure requires considerable care to prevent burns to the hands. This step reduces the space by which air and therefore humidity can reach the sample before sealing is completed.

- ***Step 13***
  Once all the vials have been constricted, place them on the ports of the lyophilizer tree, inserting the open end into each port. Thus, the end carrying the sample remains visible (**Photo III–42**). Repeat the drying process in the lyophilizer for about 1 h to eliminate any humidity that may have reached the sample during *Step 12*.

- ***Step 14***
  Without switching off the lyophilizer, seal the vials, one by one, by vacuuming and then using the propane gas torch to melt the glass and thus constrict the neck (**Photo III–43**). Once the vials are sealed, lyophilization is completed.

***Note:*** If the vacuum is lost on sealing a vial, remove this part of the vial and replace it with a new one. Wait for the vacuum to reach the point of sealing before continuing to seal the other vials.

How long a lyophilized sample can last in storage has not been formally established. However, in our laboratory, lyophilized samples have been recovered after 30 years of storage. Lyophilized samples do have a disadvantage: to recover a sample, the entire vial must be broken. Several copies of each lyophilized sample must therefore be made to maintain an adequate collection.

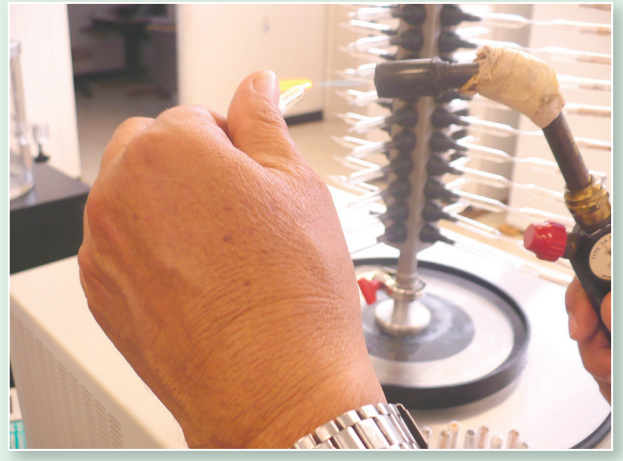
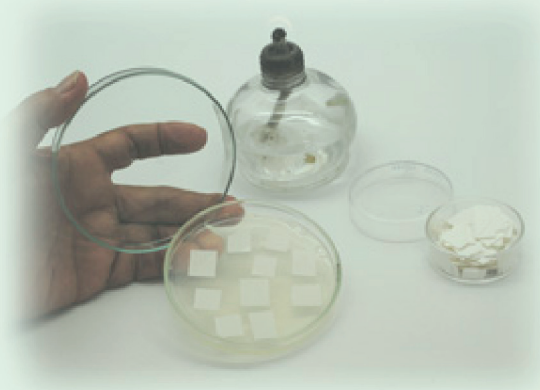


**Photo III–43**

## 2. Conservation as fungal growth on filter paper at -20 °C

This method permits long-term conservation of microorganisms.



### Materials

- Petri dishes containing PDA culture medium
- Sterilized filter paper
- Growths of *C. lindemuthianum*
- Tweezers
- Sterilized petri dishes
- Sterilized distilled water
- Pipette
- Glassine envelopes
- Scissors
- Autoclave
- Incubator
- Bunsen burner
- Marker pen

### Processing steps

- ***Step 1***
  Cut the filter paper into 60 to 80 1-cm squares. Place them in a petri dish or otherwise lidded container, and sterilize by autoclaving.

- ***Step 2***
  Place 10 of the squares in each petri dish containing PDA culture medium.

- ***Step 3***
  Prepare a suspension of conidia and mycelium of a fungal isolate, as follows:

  – Deposit sterilized distilled water on an isolate already grown in a petri dish.
  – Scrape the surface of the isolate with the pipette tip until a suspension is obtained.

- ***Step 4***
  Draw up a little of this suspension with the same pipette and place one drop on each square of sterilized filter paper (see *Step 2* above).

- ***Step 5***
  Incubate the dishes at 20 °C for 12 days.

- ***Step 6***
  After 12 days of incubation, remove the squares from the petri dishes, using sterilized tweezers and, complying with all conditions of asepsis and sterilization, deposit them in an empty and sterilized petri dish. Place the squares so that the fungus faces downwards. This should ensure that the papers will not curl on drying. Dry the squares at 24 °C for 7 days.

- ***Step 7***
  Use flame-sterilized tweezers to transfer the dried paper squares to small and sterilized glassine envelopes. Introduce these into either a larger glassine bag or small box suitable for storage (**Photos III–47** and **III–48**). Write information on the fungus on this large bag (or box) and store at -20 °C.

## 3. *Conservation as a suspension on filter paper at -20 °C*

This is another storage method that could be used if infrastructure for lyophilization is not available. The methodology is used to conserve a pathogen in suspension in either peptone-sucrose or peptone-dextrose solution, deposited on pieces of filter paper for storage at -20 °C.

### Materials (Photo III–44)

- Peptone-sucrose or peptone-dextrose solution, prepared as described in *III.F.1., Step 5*
- Petri dishes containing sporulating fungal cultures
- Petri dishes containing PDA culture medium
- Tweezers
- Spatula
- Pipettes
- Filter paper
- Marker pen
- Glassine envelopes
- Petri dishes

Photo III–44

## Processing steps

- ***Step 1***
  Take a petri dish containing a sporulated fungal strain and add 2 mL of peptone-sucrose solution. (**Photo III–45**).

- ***Step 2***
  Use a sterilized spatula (or the same pipette used to add the solution) to scrape the surface of the fungus, thus detaching conidia and obtaining a conidial suspension.

- ***Step 3***
  Use sterilized tweezers to place twenty 0.5-cm squares of sterilized filter paper in a petri dish containing PDA culture medium and impregnate them with the fungus suspended in peptone-sucrose solution (**Photo III–46**).

- ***Step 4***
  Use sterilized tweezers to remove the squares of filter paper from the petri dish and place them on filter paper lining the bottom of another, sterilized petri dish. Dry at 24 °C for 7 days.

**Photo III–45**

**Photo III–46**

Photo III–47

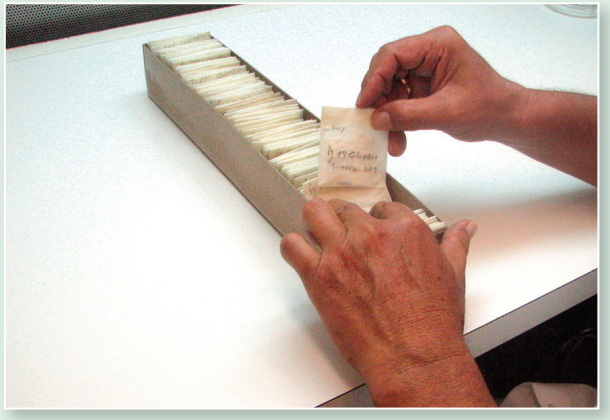


Photo III–48

- ***Step 5***
  After drying, place the squares in sterilized glassine envelopes (**Photo III–47**) for storage. Write on the envelopes information on the fungus such as storage date and strain name (**Photo III–48**). Store at -20 °C.

## G. Proof of storage

The purpose of this test is to guarantee that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.

### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with the fungal suspension and dried for 7 days at 24 °C.

- ***Step 2***
  Culture the square on PDA medium. After 5 days, the pathogen should be growing on the medium.

- ***Step 3***
  Examine the fungal growth under the stereoscope.
  If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

# H. Recovering stored fungus

## *1. Conserved by lyophilization*

### Materials (Photo III–49)

- Peptone-sucrose solution, prepared as described in ***III.F.1., Step 5***
- Petri dishes containing PDA culture medium
- Bulb pipette
- Pestle
- Sterilized cloth napkin
- Tweezers
- Incubator

### Processing steps

- ***Step 1***
  Select a vial containing the lyophilized fungus to be restored. Break the vial by tapping it with a pestle on the end opposite to that carrying the fungus (**Photo III–50**). Use the cloth napkin to cushion the blows and contain the breaking glass. Remove the cotton wool plug from inside the vial, using tweezers (**Photo III–51**).

**Photo III–49**

**Photo III–50**

**Photo III–51**

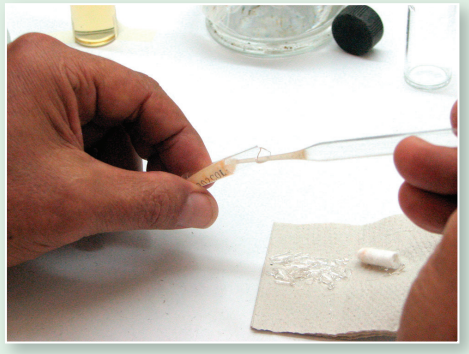


Photo III–52

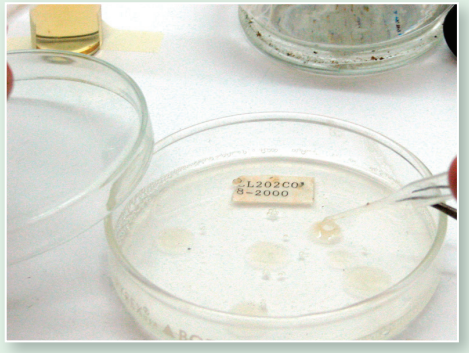


Photo III–53

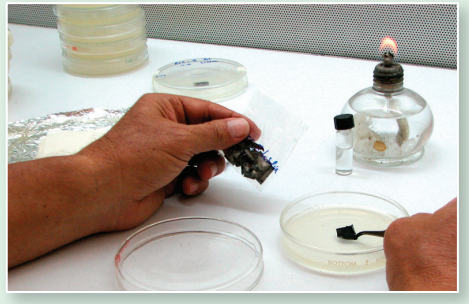


Photo III–54

- ***Step 2***
  With a pipette, deposit 4 drops of peptone-sucrose solution in the open half of the vial. The lyophilized fungal cells will become suspended in the solution and can then be removed from the vial (**Photo III–52**).

- ***Step 3***
  Culture the fungal suspension into a petri dish containing PDA medium to reinitiate fungal growth (**Photo III–53**).

## *2. Conserved on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (**see *III.F.2., Step 6***) containing squares of filter paper carrying the fungal isolated to be restored. Use sterilized tweezers to select a square and transfer it to a petri dish (**Photo III–54**) containing PDA culture medium.

- ***Step 2***
  Use a pipette to deposit 1 or 2 drops of peptone-sucrose solution on each selected square, thus hydrating the fungus. Then use the tip of the same pipette to spread the fungus over the medium's surface (**Photo III–55**). This will increase the area over which the fungus will grow.

- ***Step 3***
  Incubate the petri dishes at 20 °C. Fungal growth will reactivate and, after 12 days, the fungus will be ready for use in later procedures.

## *3. Conserved as a suspension on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (**see *III.F.3., Step 5***) containing squares of filter paper carrying the fungal strain to be restored. Remove 1 or 2 squares and transfer them to petri dishes containing PDA culture medium.

- ***Step 2***
  Incubate the petri dishes at 20 °C. After 2 or 3 days, fungal growth should be seen. Eight days after incubation, the fungus should be ready for multiplication.

**Photo III–55**

# Practical Guide 4

*Fusarium oxysporum*

(Fusarium wilt, fusarium yellows)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
*Since 1967 / Science to cultivate change*

## Contents

# Considerations

Photo IV–1

Photo IV–2

The fungus *Fusarium oxysporum* f. sp. *phaseoli* Schlecht. *emend.* Snyder & Hansen causes the plant disease known as fusarium wilt or fusarium yellows. The fungus penetrates plants through their roots, then invades xylem vessels, and soon plugs the entire vascular system. The first symptom is plant yellowing; wilting is later observed as a consequence of vascular system destruction. The plant then suffers defoliation and eventually dies. (**Photos IV–1** and **IV–2**).

# Procedures

## A. Collecting and shipping samples

To isolate the fungus *Fusarium oxysporum*, first collect bean plants showing typical symptoms of the disease on roots and hypocotyls—the two structures that the fungus usually attacks.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying samples
- Marker pen and fieldbook

- ***Paper towels.*** Wrap samples of diseased bean roots or stems in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the diseased tissue, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach the labels to their corresponding samples.

## *Recommendations*

– IIndicate on the label if collection was in a farmer's field or on an experimental station.
– If there are not enough labels for all the samples, code the bags and record the full information in a fieldbook.
– Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once at the laboratory, and if they are not to be processed immediately, leave them out to finish drying on paper towels spread on a bench.

### Processing steps

- ***Step 1***
  Take samples of roots or stems from plants presenting fusarium wilt.
- ***Step 2***
  Wrap each sample in a paper towel, place it in a paper bag or envelope, and firmly attach the corresponding label.
- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons mentioned above.

## B. Preparing PDA culture medium

The letters of the abbreviation "PDA" correspond to components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- Dehydrated PDA at 39 g/L
- 1 L distilled water
- Two 1000-mL erlenmeyer flasks
- 50 petri dishes
- Large receptacle (e.g., a beaker)
- Balance
- Autoclave

Photo IV–3

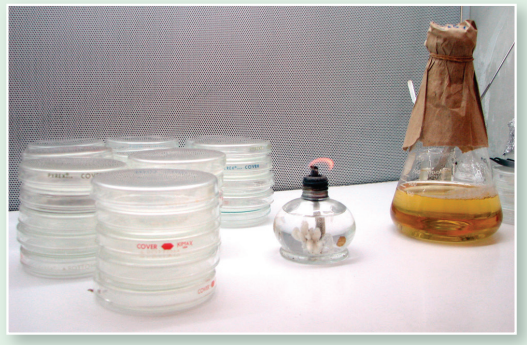

Photo IV–4

Photo IV–5

### Preparation

- Weigh the ingredients (**Photo IV–3**), place them in a large receptacle such as a beaker, and add water. Pour the resulting solution (PDA culture medium) into two erlenmeyer flasks at 500 mL each.
- Sterilize the flasks containing the medium by autoclaving for 40 min at 121 °C and a pressure of 20 lpsi.
- Leave the sterilized medium until cool enough (**Photo IV–4**) to pour into petri dishes at 20 mL per dish (**Photo IV–5**).

## C. Isolating *F. oxysporum*

### Materials

The fungus can be isolated on PDA medium using the following materials :

- Samples of diseased plant tissues
- Petri dishes containing PDA culture medium
- Laminar-flow chamber
- Scissors
- 60- and 100-mm petri dishes
- Sterilized distilled water
- 2.5% sodium hypochlorite
- Paper towels
- Bunsen burner
- Incubator
- Tweezers
- Stereoscope

- Needle
- Bent-glass streaking rod
- Pipettes

***Note:*** All the procedures must be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

## *1. On PDA culture medium*

### Processing steps

- ***Step 1***
  Use sterilized scissors to cut diseased tissue into several pieces (**Photo IV–6**).

- ***Step 2***
  Disinfect the sample pieces for 3 min in a 60-mm petri dish containing 2.5% sodium hypochlorite. Then take up another petri dish and rinse the disinfected pieces with sterilized distilled water, using a sterilized pipette. (**Photo IV–7**).

- ***Step 3***
  Place the disinfected and washed pieces on sterilized paper towels to dry for 10 min (**Photo IV–8**).

**Photo IV–6**

**Photo IV–7**

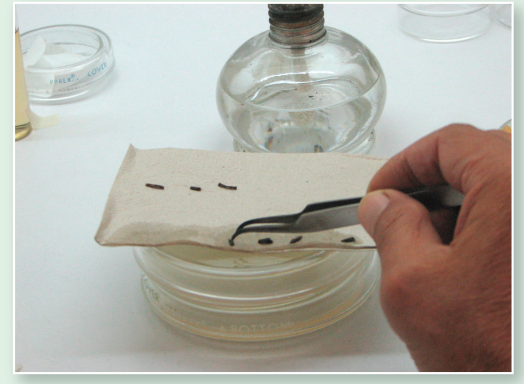

**Photo IV–8**

Photo IV–9

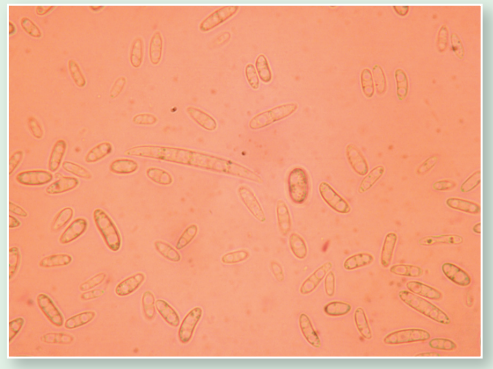

Photo IV–10

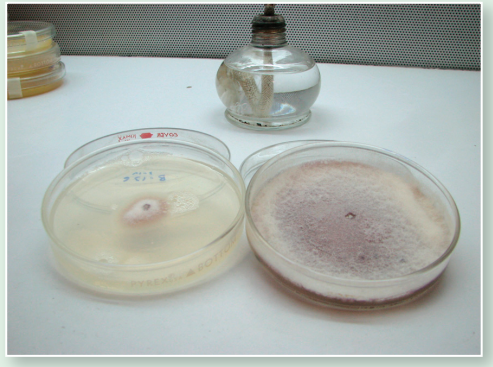

Photo IV–11

- ***Step 4***
  Once the pieces are dry, use sterilized tweezers to select a tissue piece to be placed on petri dish containing PDA medium (**Photo IV–9**). Continue with the other pieces, thereby processing all the samples.

- ***Step 5***
  Incubate the dishes at 24 °C for 7 days, by which time the fungus will have produced conidial masses and mycelium (**Photos IV–10** and **IV–11**).

## 2. As a monosporic culture

Because a sample of diseased tissue may carry a mixture of pathogenic strains, the genetic purity of an *F. oxysporum* isolate is assured only when that isolate is generated from one conidium.

Monosporic cultures are obtained from the fungal growth developed by infected tissue on PDA medium (see above, *Step 5*).

## Processing steps

- ***Step 1***
  Use a stereoscope to select a conidial mass and, with a flame-sterilized needle. Touch the conidial mass and thus pick up several conidia adhering to the needle point.

- ***Step 2***
  Over PDA culture medium in a petri dish, wash the conidia off the needle with sterilized distilled water from a pipette. That is, deposit 4 to 6 drops of water onto the needle held over the medium. Spread the water carrying the conidia over the medium's surface, using a bent-glass streaking rod.

  Ensure that the streaking rod is flame-sterilized before each use to prevent contaminating the medium.

- ***Step 3***
  Incubate the petri dishes at 20 °C for 24 h, by which time the conidia will be germinating and ready for individual transfer.

- ***Step 4***
  Examine the germinated conidia under a stereoscope and select one that is completely separated from the other ones. Remove it, using a flame-sterilized needle, and transfer it to a petri dish containing PDA culture medium.

- ***Step 5***
  Incubate the dish at 24 °C. The germinated conidium should develop massive fungal growth, and after 10 days, it completely covers the medium with the structures of a monosporic fungus.

The monosporic isolate is used where a genetically pure strain of the pathogen is needed for diverse procedures such as inoculation, DNA extraction from the mycelium, and conservation.

## D. Increasing *F. oxysporum* on PDA culture medium

The number of fungal increase (or transfers) to be made depends on the number of plants that are to be inoculated.

### Materials

– Petri dishes containing sporulating fungal cultures
– Petri dishes containing PDA culture medium
– Sterilized distilled water

- Bulb pipette
- Bent-glass streaking rod
- Incubator
- Bunsen burner

## Processing steps

- ***Step 1***
  Use a pipette to add 3 to 4 mL of sterilized distilled water to the isolate. With the same pipette, scrape the surface of the fungal culture to detach conidial masses and mycelium (**Photo IV–12**).

- ***Step 2***
  Transfer 1 mL of the fungal suspension per petri dish containing new PDA culture medium (**Photo IV–13**). Spread the suspension over the medium, using a flame-sterilized, bent-glass, streaking rod (**Photo IV–14**).

- ***Step 3***
  Incubate the petri dishes at 24 °C for 10 days.

**Photo IV–12**

**Photo IV–13**

**Photo IV–14**

# E. Inoculating plants

## Materials (Photo IV–15)

- 8-day-old bean plantlets growing in pots of sterilized sand
- Petri dishes containing PDA culture medium
- Gauze
- Humid chamber
- Spatula
- Pots containing sterilized soil
- Microscope
- Petri dishes containing fungal cultures
- Hemocytometer
- Receptacle for inoculum suspension
- Sterilized distilled water
- Running water
- Scissors
- Sterilized petri dishes
- Greenhouse table

Photo IV–15

## Processing steps

- ***Step 1***
  Ten days before inoculation, multiply the fungus according to the number of plants to be inoculated. Calculate the number of petri dishes containing PDA medium needed to make the required amount of inoculum. Then culture the fungus (**see *IV.D.* above**).

Three or four petri dishes of cultured fungus are sufficient for preparing about 500 mL of inoculum.

- ***Step 2***
  Add sterilized distilled water to the fungal growths. Then scrape the isolate's surface with a sterilized spatula to detach the conidia and thereby obtain a conidial suspension. Filter it through a piece of sterilized gauze to separate particles such as agar residues and mycelium. Collect the filtrate containing conidia in a 60-mm sterilized petri dish.


- ***Step 3***
  Use a microscope to count the conidia found in the central grid of a hemocytometer comprising one central and four outer squares. Multiply the number of conidia counted by 50,000. In this case, the concentration of the inoculum should be $1 \times 10^6$ conidia per milliliter. Given the final volume of inoculum to be sprayed ($V_2$) and given the concentration of conidia in the original inoculum, use the following formula to find the volume of the original inoculum needed for spraying:

$$V_1 \times C_1 = V_2 \times C_2$$

For example, if 125 conidia are counted in the hemocytometer, then:

$$125 \times 50.000 = 6{,}250{,}000$$
$$= 6.25 \times 10^6 \text{ (conidia in 1 mL)}$$

**Photo IV–16**

**Photo IV–17**

**Photo IV–18**

This is the concentration of the original inoculum (i.e., the filtrate obtained in *Step 2* above). If 250 mL of inoculum with a concentration of $1 \times 10^6$ conidia per milliliter is needed then, by substituting these data in the formula described above, the value of the volume of the original (also called initial) inoculum needed ($V_1$) can be found, that is:

$$V_1 \times 6.25 \times 10^6 = 250 \text{ mL} \times 1 \times 10^6$$

Thus,

$$V_1 = \frac{250 \text{ mL} \times 1 \times 10^6}{6.25 \times 10^6} = 40 \text{ mL}$$

To obtain the final concentration desired, take the 40 mL of initial inoculum and complete it to 250 mL with sterilized distilled water.

- ***Step 4***
  Carefully remove the 8-day-old bean plantlets from their pots, taking care not to damage the roots. Wash the roots with water to remove all residues of sand (**Photo IV–16**).

- ***Step 5***
  Trim the ends off the rootlets, using sterilized scissors (**Photo IV–17**), so that this mechanical injures will provide entry for the fungus, which will then initiate plant infection.

- ***Step 6***
  Submerge the cut roots for 4 min in receptacles containing the inoculum suspension at the desired concentration (i.e., at 1 x $10^6$ conidia/mL) (**Photo IV–18**).

- ***Step 7***
  Plant the bean plants again into individual pots containing sterilized soil. Ensure that the entire root system is covered (**Photo IV–19**). Water the plants, using running water. Place the inoculated pots in a humid chamber for 1 week (**Photo IV–20**) at 22 °C and relative humidity between 90% and 100%.

- ***Step 8***
  After incubation, transfer the pots to a greenhouse table at room temperature and leave for another 15 days. By this time, the plants will have presented symptoms typical of the disease and can then be evaluated. Usually, a bean plant will react as either susceptible, with plant death, or resistant, showing no symptoms. Intermediate reactions are uncommon.

**Photo IV–19**

**Photo IV–20**

# F. Conserving the fungus for storage

The fungus *F. oxysporum* can be conserved either by lyophilization or on pieces of filter paper impregnated with the fungus suspended in peptone-sucrose solution and kept in glassine envelopes at -20 °C.

## *1. Lyophilization*

### Materials (Photo IV–21)

Photo IV–21

- Tree manifold lyophilizer with two types of supports (rack and tree) and 48 ports for vials
- 0.5-mL neutral-glass vials for lyophilization, and rack
- Growths of fungus on PDA culture medium
- Filter paper
- Long Pasteur pipettes
- Tweezers or inoculation needle
- Peptone at 10%
- Distilled water
- Sucrose or dextrose at 20%
- Autoclave
- Scissors
- Receptacle
- Cotton wool
- Propane gas torch
- Freezer
- Bunsen burner
- Marker pen and notebook

***Note:*** Monosporic fungal isolates must be prepared 10 days before lyophilization (**see *IV.C.2.* above**). The fungus should be producing conidia.

## Processing steps

- ***Step 1***
  Write in either small lettering or code on 1- to 2-cm squares of filter paper to identify the isolates to be lyophilized. Include the fungus's genus and species names, isolate number (i.e., the code the laboratory uses), and storage date.

  If you write in code, write the full information in a notebook.

- ***Step 2***
  Use sterilized scissors to cut out the pieces of filter paper identifying each isolate and introduce each piece to the bottom of a vial.

- ***Step 3***
  Then prepare a cotton wool plug to stopper each vial so that it plugs the upper two thirds.
  – Tease out a piece of cotton wool until it measures about 5 x 3 cm and roll it around a tweezers arm or inoculation needle, pressing between fingers and thumb until a plug is formed (**Photos IV–22**, **IV–23**, and **IV–24**). Leave a piece of unrolled cotton wool on the end opposite the plug.

**Photo IV–22**

**Photo IV–23**

**Photo IV–24**

Photo IV–25

- Introduce the plug into the vial and gently withdraw the tweezers arm or needle with one hand while holding the plug in the vial with the other.
- Prepare additional vials with their respective plugs to have spares on hand as needed. If a plug disintegrates on handling, it can therefore be replaced by one from the additional vials.

- ***Step 4***
  Autoclave all the vials with their respective plugs for 40 min until sterilized.

- ***Step 5***
  **Preparing peptone and sucrose solutions.**
  Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a 20% sucrose solution by dissolving 20 g of sucrose in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix them in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose (or peptone-dextrose) solution.

- ***Step 6***
  Draw up 2 to 3 mL of the peptone-sucrose solution, using a long Pasteur pipette and deposit them on a fungal culture (**Photo IV–25**). Homogenize the fungus within the peptone-sucrose solution by first scraping the culture with the same Pasteur pipette to detach mycelium and conidia. Then repeatedly draw up and expel the mixture until homogenized.

- ***Step 7***
  Use the same pipette from Step 6 to draw up 2 to 3 mL of the homogenized suspension (**Photos IV–26** and **IV–27**) and deposit them inside a vial, as follows:

  – In one hand take the Pasteur pipette and, in the other, the vial with its respective plug as prepared in *Step 3* above.
  – Hold the Pasteur pipette between index finger and thumb, and use the little finger (or pinky) of the same hand to hold the loose end of the cotton wool plug against the palm. Carefully withdraw the plug from the vial, keeping it firmly against the palm. Meanwhile insert the pipette into the vial and deposit the fungal suspension on the bottom, which should have the piece of filter paper identifying the isolate.
  – Stopper the vial by gently introducing the plug, which had been held between the little finger and palm (**Photo IV–28**), back into the vial.

  If the cotton wool plug comes into contact with another surface or other substance, it becomes contaminated. Discard it therefore and replace it with one of the additional plugs that had been prepared in *Step 3* for such an eventuality.

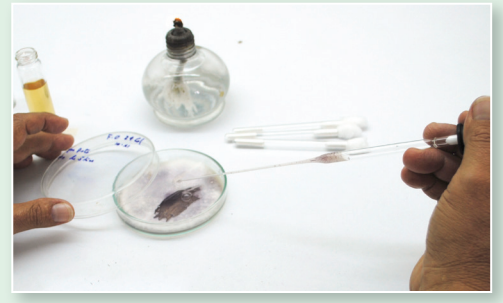

**Photo IV–26**

**Photo IV–27**

**Photo IV–28**

Photo IV–29

Photo IV–30

Photo IV–31

- ***Step 8***
  Cut off that part of the plug remaining outside the vial, using flame-sterilized scissors (**Photo IV–29**). Then introduce the rest of the plug into the vial, using the point of a scissors arm, until 1 cm is left between the lip of the vial and the plug (**Photo IV–30**).

- ***Step 9***
  Place all the prepared vials in a rack. Then push the cotton wool plug to the bottom of each vial until it touches the piece of filter paper identifying the isolate. To do this, use a long object such as an inoculation needle, flame-sterilizing it constantly to prevent contaminating the plugs (**Photo IV–31**).

  The upper part of the vial, which has no plug, is sealed after lyophilization (see *Step 14* below).

- ***Step 10***
  Once the vials are organized, freeze them at 0 °C for 15 min in the lyophiliser. When the samples are frozen, lyophilization is initiated.

- ***Step 11***
  Remove the rack from the freezing step and place it under the lyophilizer dome. Switch on the machine (**Photo IV–32**). The temperature inside the apparatus will drop to -55 °C, at which point a vacuum pump will begin extracting water, drying the samples. This process takes 20 to 22 h to complete.

- ***Step 12***
  The next day, switch off the lyophilizer and remove the rack of vials. Prepare a propane gas torch for constricting the vials in the part opposite the sample. That is, make a neck in each vial by softening the glass with the torch's flame and pulling gently on the ends of the vial. (**Photos IV–33** and **IV–34**).

  This procedure needs considerable care to prevent burns to the hands. This step reduces the space by which air and therefore humidity can reach the sample before sealing is completed.

- ***Step 13***
  Once all the vials have been constricted, place them on the ports of the lyophilizer tree, inserting the open end into each port. Thus, the end carrying the sample remains visible (**Photo IV–35**). Repeat the drying process in the lyophilizer for about 1 h to eliminate any humidity that may have reached the samples during *Step 12*.

**Photo IV–32**

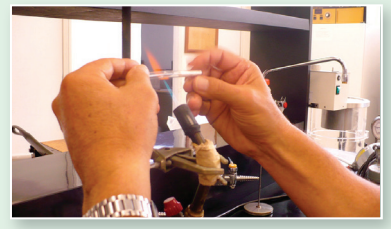

**Photo IV–33**

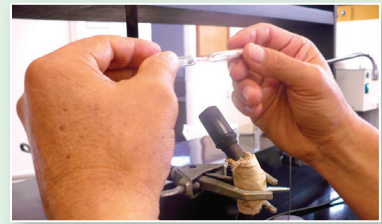

**Photo IV–34**

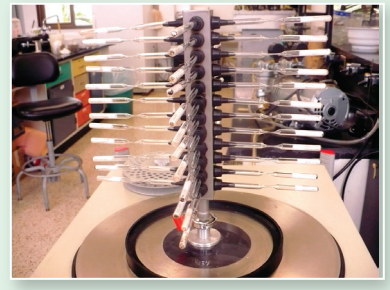

**Photo IV–35**

Photo IV–36

- ***Step 14***
  Without switching off the lyophilizer, seal the vials, one by one, by vacuuming and then using the propane gas torch to melt the glass and constrict the neck (**Photo IV–36**). Once the vials are sealed, lyophilization is completed.

***Note:*** If the vacuum is lost on sealing a vial, remove this part of the vial and replace it with a new one. Wait for the vacuum to reach the point of sealing before continuing to seal the other vials.

How long a lyophilized sample can last in storage has not been formally established. However, in our laboratory, lyophilized samples have been recovered after 30 years of storage. Lyophilized samples do have a disadvantage: to recover a sample, the entire vial must be broken. Several copies of each lyophilized sample must therefore be made to maintain an adequate collection.

## 2. Conservation as fungal growth on filter paper at -20 °C

This method permits long-term conservation of microorganisms.

### Materials

- Petri dishes containing PDA culture medium
- Growths of *F. oxysporum*
- Sterilized petri dishes
- Incubator
- Sterilized distilled water
- Autoclave
- Pipette
- Bunsen burner
- Glassine envelopes
- Scissors
- Marker pen
- Tweezers
- Filter paper

### Processing steps

- ***Step 1***
  Cut the filter paper into 60 to 80 1-cm squares, place them in a petri dish or lidded container, and sterilize by autoclaving.

- ***Step 2***
  Place 10 of the squares in each petri dish containing PDA culture medium.

- ***Step 3***
  Prepare a suspension of conidia and mycelium from a fungal isolate, as follows:

  – Deposit sterilized distilled water on an isolate already grown in a petri dish.
  – Scrape the surface of the culture with the tip of a pipette until a suspension is obtained.
  – Draw up a little of this suspension with the same pipette and place one drop on each square of sterilized filter paper (*Step 2* above).

***Note:*** An alternative step is to place a similar-sized piece of PDA medium on each square of filter paper. Use the same PDA medium on which the fungus had been growing as a monosporic culture (**see *M.C.2.* above**). Then deposit a drop of the fungal suspension described in *Step 3* above.

- ***Step 4***
  Incubate the dishes at 24 °C for 10 days.

- ***Step 5***
  After the 10 days, remove the squares of filter paper from the petri dishes, using sterilized tweezers and, complying with all conditions for asepsis and sterilization, deposit them in an empty, sterilized petri dish. Place the squares

so that the fungus faces downwards. This should ensure that the papers will not curl on drying. Dry the papers at 24 °C for 7 days.

- ***Step 6***
  Use flame-sterilized tweezers to transfer the dried squares to sterilized glassine envelopes. Introduce these into either a larger glassine bag or small box suitable for storage (**Photos IV–40** and **IV–41**). Write the information on the fungus on this large bag or box, and store at -20 °C.

## 3. Conservation as a suspension on filter paper at -20 °C

### Materials

- Peptone-sucrose solution, prepared as described in ***IV.F.1, Step 5***
- Petri dishes containing sporulating fungal growths
- Autoclave
- Spatula
- Receptacle with lid
- Filter paper
- Petri dishes
- Scissors
- Pipettes
- Marker pen
- Tweezers
- Glassine envelopes
- Freezer

Photo IV–37

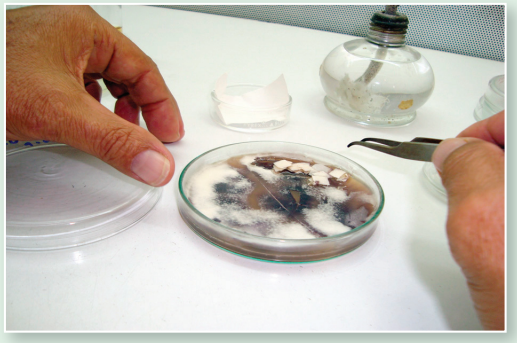

Photo IV–38

Photo IV–39

## Processing steps

- ***Step 1***
  Cut several 0.5-cm squares of filter paper, place them in a lidded receptacle, and sterilize them by autoclaving.

- ***Step 2***
  Take a petri dish containing a sporulated fungal isolate and add 2 mL of peptone-sucrose solution (**Photo IV–37**).

- ***Step 3***
  Use a sterilized spatula (or the same pipette used to add the solution) to scrape the surface of the fungus, detaching conidia and thus obtaining a conidial suspension.

- ***Step 4***
  Use sterilized tweezers to place 20 squares of filter paper in the petri dish from *Step 3* above and impregnate them with the suspension of fungus and peptone-sucrose solution (**Photo IV–38**).

- ***Step 5***
  Use sterilized tweezers to remove the squares and place them on filter paper lining the bottom of another, sterilized petri dish. Dry at 24 °C for 7 days (**Photo IV–39**).

- ***Step 6***
  After drying, place the squares in sterilized glassine envelopes (**Photo IV–40**) for storage. Write on the envelopes information on the fungal isolate such as its name, place of origin, and storage date (**Photo IV–41**). Place the envelopes correctly into the freezer and store at -20 °C.

## G. Proof of storage

The purpose of this test is to guarantee that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.

### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with the fungal suspension and dried for 7 days at 24 °C.

- ***Step 2***
  Culture the square on fresh PDA medium. After 5 days, the pathogen should be growing on the medium. (**Photo IV–42**).

**Photo IV–40**

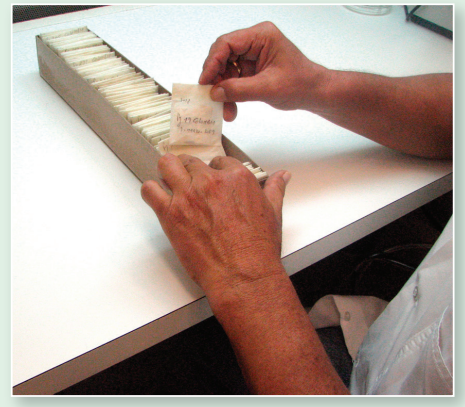

**Photo IV–41**

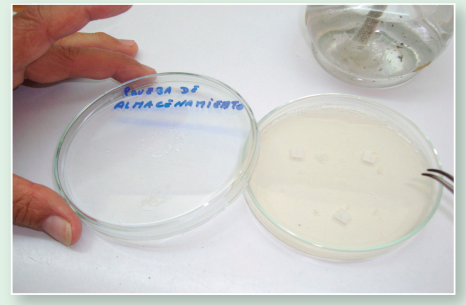

**Photo IV–42**

- ***Step 3***
  Examine the growth under the stereoscope. If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

## H. Recovering stored fungus

### Materials (Photo IV–43)

The materials needed to recover stored fungus from any of the three conservation methods include:

- Peptone-sucrose solution, prepared as described in ***IV.F.1, Step 5***
- Petri dishes containing PDA culture medium
- Bulb pipette
- Pestle
- Sterilized cloth napkin
- Tweezers
- Incubator

Photo IV–43

## 1. Conserved by lyophilization

- ***Step 1***
  Select a vial containing the lyophilized fungus to be restored. Break the vial by tapping it with a pestle (**Photo IV–44**) on the end opposite that carrying the lyophilized fungus. Use the cloth napkin to cushion the blows and contain the breaking glass. Remove the cotton wool plug from inside the vial (**Photo IV–45**), using tweezers.

- ***Step 2***
  In the open half of the vial, add 4 drops of peptone-sucrose solution, using a pipette. The lyophilized cells of the fungus will then be suspended in the solution and can be removed from the vial (**Photo IV–46**).

- ***Step 3***
  To restart growth in the fungus, culture the conidial suspension on PDA medium in a petri dish. (**Photo IV–47**).

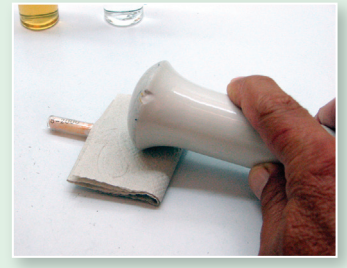

**Photo IV–44**

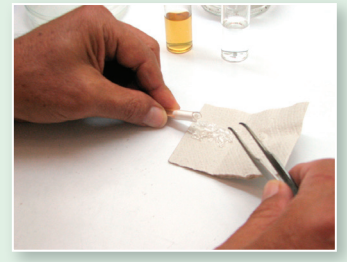

**Photo IV–45**

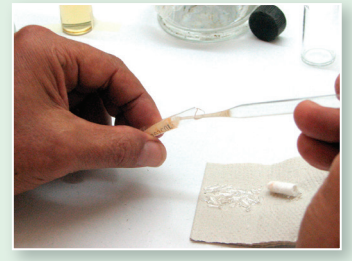

**Photo IV–46**

**Photo IV–47**

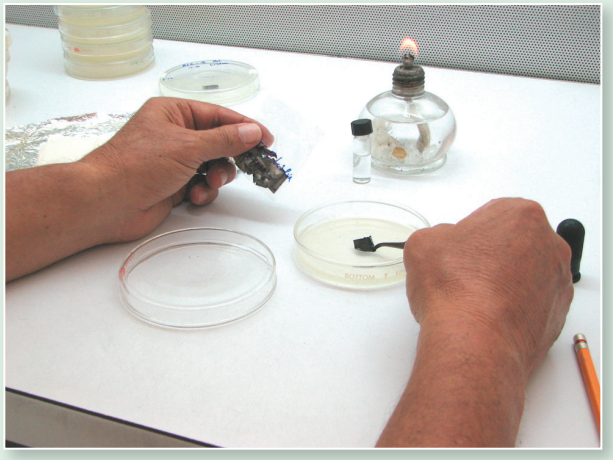

Photo IV–48

Photo IV–49

## *2. Conserved as fungal growth on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***IV.F.2., Step 6***) containing squares of filter paper carrying the fungal isolate to be restored. Use sterilized tweezers to select a square and transfer it to a petri dish containing PDA culture medium (**Photo IV–48**).

- ***Step 2***
  Use a pipette to deposit 1 or 2 drops of peptone-sucrose solution on each square, thus hydrating the fungus. Then use the tip of the same pipette to spread the solution over the entire surface of the medium (**Photo IV–49**). This will increase the area over which the fungus will grow.

- ***Step 3***
  Incubate the petri dishes at 24 °C. Fungal growth will reactivate and, after 12 days, the fungus will be ready for use in later procedures.

## 3. Conserved as a suspension on filter paper at -20 °C

### Processing steps

- ***Step 1***
  Select a glassine envelope (***IV.F.3, Step 6***) containing squares of filter paper carrying the fungal isolate to be restored. Remove 2 or 3 squares and transfer them to petri dishes containing PDA culture medium.

- ***Step 2***
  Incubate the petri dishes at 24 °C. After 2 or 3 days, the fungus will have reinitiated growth and be ready for further uses.

# Practical Guide 5

*Macrophomina phaseolina*

(Charcoal rot, charcoal root rot,
ashy stem blight)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
*Since 1967 / Science to cultivate change*

## Contents

# Considerations

Photo V–1

Photo V–2

The fungus *Macrophomina phaseolina* (Tass) Goidanich causes the plant disease known as charcoal rot, charcoal root rot, or ashy stem blight. It attacks crops of beans, soybeans, maize, sorghum, and alfalfa. It is commonly found infecting both plantlets and adult plants of bean crops suffering stress from drought and high temperatures.

- In **plantlets**, the first symptoms appear at the cotyledons (**Photo V–1**). The pathogen advances towards the root neck, killing the plantlet.

- In the field, **adult plants** show ashy grey lesions on stems, petioles, and pods. In these lesions is common to find the fungus's survival structures known as pycnidia (**Photo V–2**).

In beans, the fungus could be transmitted by contaminated seeds.

# Procedures

## A. Collecting and shipping samples

To isolate the *M. phaseolina* fungus, first collect bean tissues showing typical symptoms of the disease.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels to identify samples
- Marker pen

- ***Paper towels.*** Wrap samples of diseased bean pods or stems in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples of diseased tissue, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach labels to their corresponding samples.

## *Recommendations*

Do not collect wet plant materials. If this is unavoidable before shipping, dry them with paper towels. Once in the laboratory, and if they are not to be immediately processed, leave them out to finish drying on paper towels spread on a bench.

## Processing steps

- ***Step 1***
  Take samples of pods or stems from plants showing charcoal rot symptoms.

- ***Step 2***
  Wrap each sample in absorbent paper, place it in a paper bag or envelopes, and firmly attach the corresponding label, as described above.

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrap them in aluminum foil for the reasons explained above.

## B. Preparing PDA culture medium

The letters in the abbreviation "PDA" correspond to the components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- Dehydrated PDA at 39 g/L
- 1 L distilled water
- Two 1000-mL erlenmeyer flasks
- Balance
- Large receptacle (e.g., beaker)
- Autoclave
- Petri dishes

### Preparation

- Weigh the ingredients (**Photo V–3**), place them in a large receptacle such as a beaker, and add water. Pour the resulting solution (PDA culture medium) into two erlenmeyer flasks at 500 mL each.
- Autoclave the flasks containing the medium for 40 min at 121 °C and a pressure of 20 psi until sterilized.
- Leave the sterilized medium until cool enough (**Photo V–4**) to pour in petri dishes at 25 mL per dish (**Photo V–5**).

**Photo V–3**

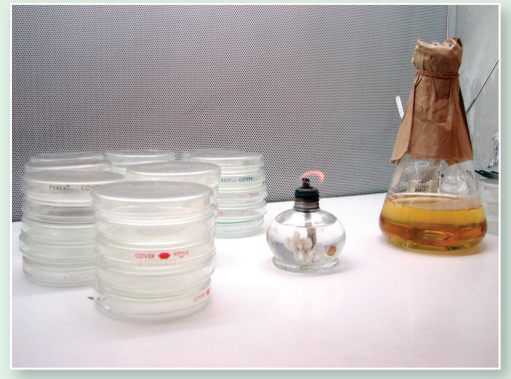


**Photo V–4**

**Photo V–5**

Photo V–6

Photo V–7

## C. Isolating *M. phaseolina*

The protocol for isolating *M. phaseolina* is simple because the tissues that the pathogen colonizes usually harbor few or no saprophytes, thus facilitating isolation and purification.

***Note:*** All procedures should be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

### *1. On PDA culture medium*

#### Materials

- Samples of diseased plant tissues
- Petri dishes containing PDA culture medium
- Scissors
- Sterilized tweezers
- 2.5% sodium hypochlorite
- Laminar-flow chamber
- Sterilized distilled water
- Incubator
- Sterilized paper towels
- Microscope or stereoscope

## Processing steps

- ***Step 1***
  Cut diseased tissue into several pieces, using sterilized scissors (**Photo V–6**).

- ***Step 2***
  Disinfect the sample pieces for 3 min in a solution of 2.5% sodium hypochlorite (**Photo V–7**). Then rinse the disinfected sample pieces three times with sterilized distilled water.

- ***Step 3***
  Place the disinfected and washed sample pieces on sterilized paper towels to dry for 10 min (**Photo V–8**).

- ***Step 4***
  Once the pieces are dry, use sterilized tweezers to select 3 pieces and culture them in petri dish containing PDA medium (**Photo V–9**). Repeat the procedure for all the samples.

- ***Step 5***
  Incubate the dishes of all the samples at 28 °C for 7 days, by which time the fungus should be producing mycelium (**Photo V–10**) and pycnidia. Under the microscope or stereoscope, the latter structure appears as numerous black spots. In 8 days, the isolated pathogen should be ready for monohyphal culture purification and multiplication to produce enough inoculum for greenhouse and field tests.

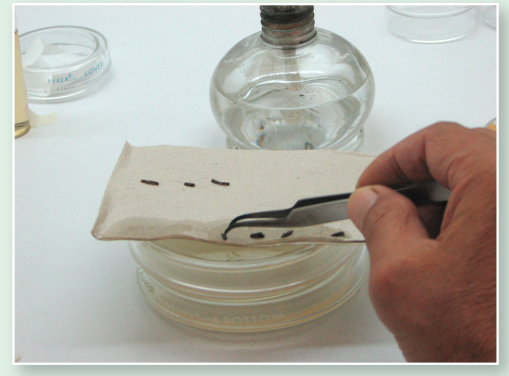


**Photo V–8**

**Photo V–9**

## 2. As a monomycelial isolate

Because a sample of diseased plant tissue may carry a mixture of pathogenic strains, the fungus needs to be purified. A genetically pure strain of *M. phaseolina* can be obtained through monomycelial isolation, using hyphal tips. This procedure is used for fungi that do not produce structures like spores or conidia.

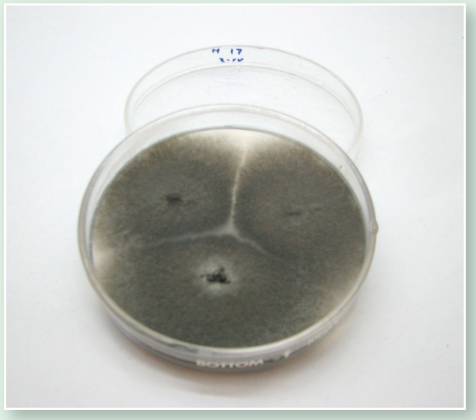


Photo V–10

### Processing steps

- ***Step 1***
  Remove a 0.5-cm square of PDA culture medium on which the fungus is growing (see *Step 5* above) and transfer the square to the center of a petri dish containing fresh PDA medium.

- ***Step 2***
  Incubate at 24 °C for 24 h. The next day, examine the fungus under the stereoscope in a laminar-flow chamber for mycelial growth. Focus on the edges of the colony to identify growing hyphal tips.

- ***Step 2***
  Once hyphal tips are identified, use a flame-sterilized needle to cut out a piece of agar carrying a single tip of one of a hyphy, as follows:

  – Take care not to touch other hyphae surrounding the selected tip.

– Transfer the piece of agar carrying the hyphal tip to another petri dish containing fresh PDA culture medium. Continue with other dishes, placing only one hyphal tip per dish.
– Incubate all the dishes at 24 °C for 10 days. By this time, a culture should be obtained that is similar to the one described in *Step 5* above, except that it is genetically pure. The fungus can then be increased for multiple uses as storage, or inoculation either in the greenhouse or field.

## D. Increasing *M. phaseolina* on white rice

### Materials (Photo V–11)

– *M. phaseolina* cultures on PDA medium
– White rice
– Aluminum foil
– Sterilized distilled water
– Kraft paper
– 15-cm-diameter petri dishes
– Autoclave
– Running water
– Incubator
– Bulb pipette

***Note:*** One petri dish will produce enough inoculum for about 40 kg of soil.

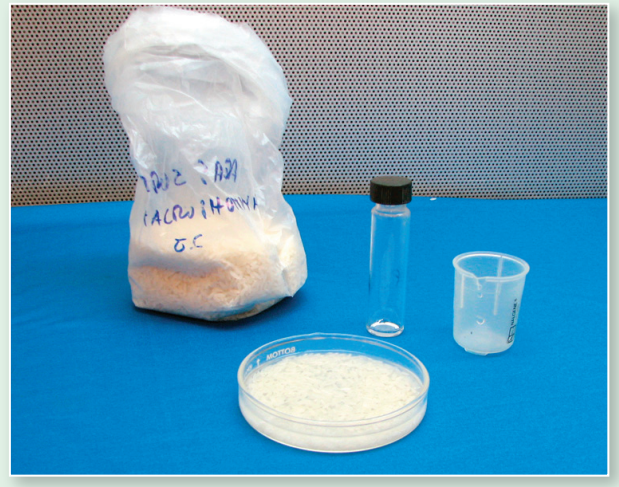


Photo V–11

Photo V–12

Photo V–13

## Processing steps

- ***Step 1***
  Place 50 g of white rice in a 15-cm-diameter petri dish and wash it once under running water.

- ***Step 2***
  Once the rice is washed, add 50 mL of running water and wrap the dish first with aluminum foil and then with kraft paper.

- ***Step 3***
  Autoclave the whole two times. After autoclaving let the sterilized rice to cool down.

- ***Step 4***
  Make a suspension of the cultured fungus (see ***V.C.2. above***) in sterilized distilled water. With a bulb pipette, draw up the water and resuspend the fungus. Then draw up the fungus suspension again with the same pipette and place 15 drops in the petri dish containing the sterilized rice (**Photo V–12**).

- ***Step 5***
  Incubate the petri dishes containing inoculated rice at 28 °C for 15 days under total darkness. During this time, the fungus will colonize the rice until it becomes black with fungal pycnidia (**Photo V–13**). The color indicates the pathogen's successful growth. If the culture shows a different color to that in the photo, then it is contaminated and must be discarded.

Remove the lids from the petri dishes containing the fungus and leave them for 1 day inside the same incubator. Excess moisture will dry off, facilitating the maceration of the fungus when preparing the inoculum.

# E. Inoculating plants

## *1. Preparing the inoculum*

The fungus cultured on white rice is used as inoculum.

### Processing steps

- ***Step 1***
  After 15 days of incubation and the day of drying (see *Step 5* above), cut up the rice colonized by fungus (**Photo V–14**). Then, with a pestle and mortar, gently mash the whole (**Photo V–15**) until a fine macerate is obtained.

  If the original pieces are too moist, place paper towels over them and apply pressure with the fingers so that the towels absorb the excess water. Leave the pieces to dry for 24 h before macerating.

- ***Step 2***
  Spread the macerate on another paper towel (**Photo V–16**) and dry it in an incubator at 28 °C for 24 h.

**Photo V–14**

**Photo V–15**

**Photo V–16**

Photo V–17

Photo V–18

- ***Step 3***
  When the macerate is completely dry, macerate it again, using a mortar and pestle, until it becomes a fine black powder (**Photo V–17**).

  This powder can be used as inoculum for pathogenicity tests of the fungus or to characterize the resistance or susceptibility reactions of bean germplasm.

## 2. *Inoculation in the greenhouse*

### Materials

- Fungal inoculum (**see *V.E.1.***)
- Bean seeds
- Sterilized soil
- Planting pots, trays, or receptacles
- Balance

***Note:*** Weigh the quantity of inoculum to be used in the greenhouse.

### Processing steps

- ***Step 1***
  Mix inoculum and soil at a constant rate of 0.5 g inoculum per 1 kg of soil. With the mixture, fill the pots, trays, or receptacles to be used in the inoculation test (**Photo V–18**).

- ***Step 2***
  Select the seeds of the targeted bean genotypes and plant them in the pots.

- ***Step 3***
  Conduct the first evaluation 14 days after inoculation. If the bean seed's genotype is highly susceptible, it will not germinate. Take this reaction into account in the evaluation.

  Visually evaluate and score emerging plants according to the **CIAT standard scale** as presented in **Photos V–19** to **V–27**, where scores:

  1 to 3 indicate a resistant plant (**Photos V–19**, **V–20**, and **V–21**)
  4 to 6 indicate a plant with an intermediate reaction (**Photos V–22**, **V–23**, and **V–24**)
  7 to 9 indicate a susceptible plant (**Photos V–25**, **V–26**, and **V–27**)

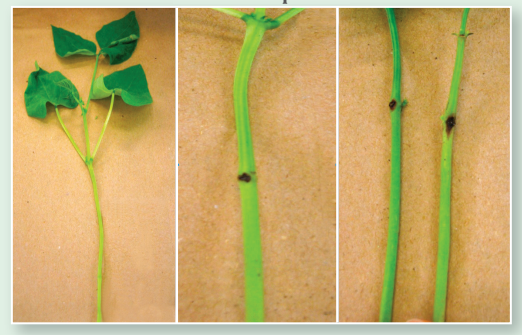
## F. Conserving the fungus for storage

The *M. phaseolina* fungus can be conserved either on filter paper at -20 °C.

### *1. Conservation as fungal growth on filter paper at -20 °C*

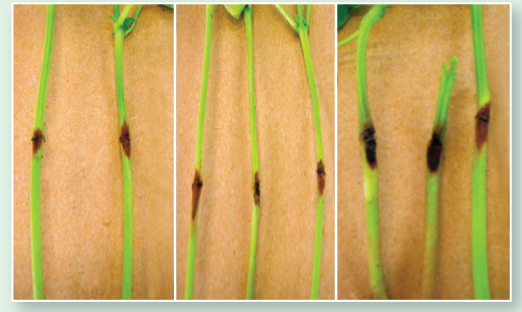
This method permits the long-term conservation of microorganisms.

Resistant plants



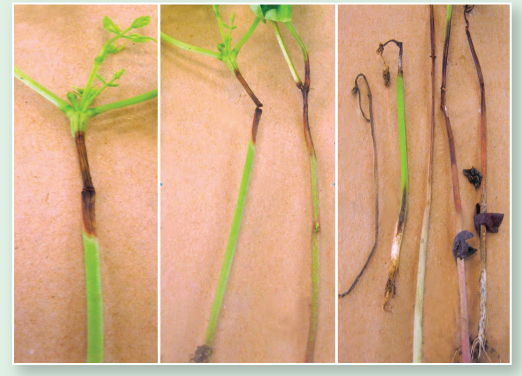
**Photos V–19, V–20, V–21**

Intermediate reaction

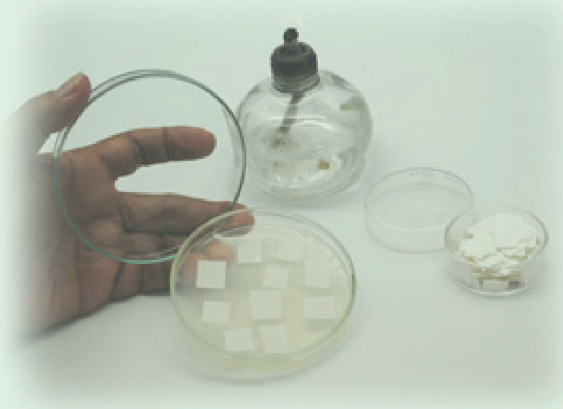


**Photos V–22, V–23, V–24**

Susceptible plants



**Photos V–25, V–26, V–27**



## Materials

- Filter paper cut into squares
- 10-day-old *M. phaseolina* growths on PDA culture medium
- Tweezers
- Sterilized petri dishes
- Sterilized distilled water
- Pipette
- Sterilized glassine envelopes
- Petri dishes containing PDA medium
- Incubator
- Autoclave
- Marker pen

## Processing steps

- ***Step 1***
  Place 1-cm squares of filter paper in a petri dish or lidded container and sterilize by autoclaving.

- ***Step 2***
  Place 10 of the sterilized squares of filter paper in petri dishes containing fresh PDA culture medium at 2 or 3 squares per dish.

- ***Step 3***
  Prepare a suspension of pycnidia and mycelium from a fungal isolate, as follows:

- Add sterilized distilled water to a petri dish containing a 10-day-old *M. phaseolina* growth.
- Scrape the entire surface of the isolate with the tip of a pipette to generate a suspension.
- Draw up a little of this suspension, using the same pipette, and place a drop on each square of sterilized filter paper.

- ***Step 4***
  Incubate the petri dishes at 24 °C for 12 days.

- ***Step 5***
  After incubation, use sterilized tweezers to remove the squares of filter paper from the medium, together with the fungus growing on them. Follow all conditions for asepsis and sterilization, and deposit them upside down in an empty, sterilized petri dish. The fungus should face downwards so that it does not curl on drying. Dry the squares at 24 °C for 7 days.

- ***Step 6***
  Use sterilized tweezers to transfer the dried squares to sterilized glassine envelopes, which are, in their turn, introduced into a larger glassine bag. Write on the envelopes information corresponding to their respective fungus such as storage date and strain name. Store the fungus at -20 °C.

Photo V–28

Photo V–29

## 2. *Conservation as a suspension on filter paper at -20 °C*

### Materials (Photo V–28)

- Petri dishes containing 10-day-old fungus
- Peptone at 10%
- Glassine envelopes
- Sucrose at 20%
- Marker pen
- Tweezers
- Distilled water
- Spatula
- Autoclave
- Sterilized filter paper
- Receptacle
- Sterilized petri dishes

### Preparing the peptone and sucrose solutions

Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a 20% sucrose solution by dissolving 20 g of sucrose in 100 mL of distilled water. Autoclave the two solutions. Once sterilized, mix the solutions in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose solution.

## Processing steps

- ***Step 1***
  Take a petri dish containing the fungus and add 2 mL of peptone-sucrose solution (**Photo V–29**).

- ***Step 2***
  Use a sterilized spatula to scrape the surface of the fungus to detach pycnidia and mycelium (**Photo V–30**). Leave the suspension in the same petri dish.

- ***Step 3***
  Place 0.5-cm squares of filter paper in the fungal suspension. Ensure that they are well impregnated (**Photo V–30**).

- ***Step 4***
  Use sterilized tweezers to remove the squares and place them on filter paper lining the bottom of another, sterilized petri dish. Dry them at 24 °C for 7 days.

- ***Step 5***
  Once dried, place the squares in sterilized glassine envelopes (**Photo V–31**) for storage. Write on the envelopes the information corresponding to the fungus such as storage date and strain name (**Photo V–32**). Store at 20 °C

**Photo V–30**

**Photo V–31**

**Photo V–32**

## G. Proof of storage

The purpose of this test is to guarantee that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.

### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with *M. phaseolina* suspension and dried for 7 days.

- ***Step 2***
  Culture the square on PDA medium. After 3 days, the pathogen should be growing on the medium.

- ***Step 3***
  Examine the fungal growth under the stereoscope.
  If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

# H. Recovering stored fungus

## *1. Conserved as fungal growth on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***V.F.1, Step 6***) containing squares of filter paper carrying the fungus isolate to be restored. Use sterilized tweezers to select and transfer a square to a petri dish containing PDA culture medium (**Photo V–33**).

- ***Step 2***
  Incubate the dishes at 24 °C. After 3 days, examine the pathogen's growth.

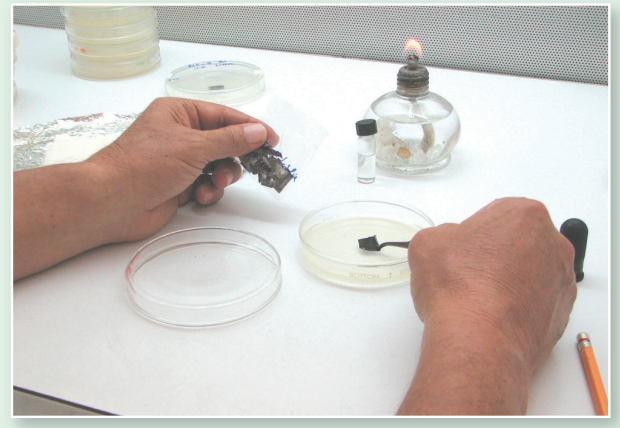


**Photo V–33**

## 2. Conserved as a suspension on filter paper at -20 °C

To reactivate *M. phaseolina* stored under this protocol, add peptone-sucrose solution to the medium used for reactivation. The solution will provide extra nutrients to the fungus, fostering its growth.

### Processing steps

- ***Step 1***
  Select a glassine envelope (***V.F.1., Step 6***) containing squares of filter paper carrying the fungal isolate to be restored. Remove 2 or 3 squares and transfer them to a petri dish containing PDA culture medium. Then add 2 drops of peptone-sucrose solution to each cultured square. Repeat the same procedure for each isolate being targeted.

- ***Step 2***
  Incubate the petri dishes at 24 °C. After 3 days, the fungus should reinitiate growth. Ten days later, it should be ready for use in different laboratory procedures such as inoculation or obtaining mycelium for DNA extraction.

# Practical Guide 6

*Phaeoisariopsis griseola*

(Angular leaf spot)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
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## Contents

# Considerations

Photo VI–1

Angular leaf spot of bean is caused by the fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris. This disease is one of the most frequently found in crops of common bean (*Phaseolus vulgaris*) grown in the tropics and subtropics.

In a highly susceptible bean variety, the initial symptoms of the disease usually appear as circular spots on the primary leaves. In the folioles of more mature leaves, the edges of the lesions are usually defined by the leaf's venation, making them appear angular and thus giving the disease its common name (**Photo VI–1**).

# Procedures

## A. Collecting and shipping samples

To isolate the fungus *Phaeoisariopsis griseola*, first collect diseased bean tissues, preferably leaves. Never collect samples from old or senescent plant tissues, as these carry saprophytes that complicate the isolation of *P. griseola*. Discard all diseased plant tissues that show signs of insect damage or other diseases.

***Note:*** The isolation of this pathogen is easier from diseased leaf tissue as different lesions appear on the same leaf. From each lesion, several isolates can also be obtained. If collecting symptomatic pods, prefer those that are green.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying samples
- Marker pen or pencil and fieldbook

- ***Paper towels.*** Once samples are removed from diseased bean leaves, pods, or stems, wrap them in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place samples of diseased tissue, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and will therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the growth of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach the labels to their corresponding samples.

## *Recommendations*

- Indicate on the label if collection was in a farmer's field or on an experimental station.
- If there are not enough labels for all the samples, code the bags and record their corresponding information in a fieldbook.
- Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once in the laboratory, and if they are not to be immediately processed, leave them out to finish drying on paper towels spread on a bench.

## Processing steps

- ***Step 1***
  Take samples of leaves, pods, or stems from plants presenting angular leaf spot (**Photo VI–2**).

- ***Step 2***
  Wrap each sample in a paper towel (**Photo VI–3**), place it in a paper bag or envelope, and firmly attach the corresponding label (see above).

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons explained above.

**Photo VI–2**

**Photo VI–3**

## B. Preparing V8 culture medium

The ideal medium for isolating and multiplying the *P. griseola* fungus has, as its major component, V8 juice, which is stocked by supermarket chains. As its brand name indicates, this juice is composed of extracts from eight vegetables: tomato, carrot, celery, beetroot, parsley, lettuce, leek, and spinach. It also contains salt, vitamin C (ascorbic acid), and citric acid. The culture medium is prepared with the following ingredients:

### Materials

- Distilled water
- 23 g agar
- 3 g calcium carbonate ($CaCO_3$)
- 1 can of V8 juice at 340 mL
- 60 petri dishes
- Three 1000-mL erlenmeyer flasks
- Balance
- Beaker
- Autoclave

### Preparation

- Weigh the ingredients, place them in a beaker, and add 1200 mL of water to bring the volume to 1500 mL.
- Mix and pour the resulting solution (V8 culture medium) in 1000-mL erlenmeyer flasks at 500 mL each (**Photo VI–4**).

**Photo VI–4**

– Autoclave the flasks for 40 min at 121 °C and a pressure of 20 psi until sterilized.
– Leave the flasks until the medium is cool enough to pour into 60 petri dishes at 20 mL per dish.
(**Photos VI–5** and **VI–6**).

## C. Isolating *P. griseola* as a monosporic culture

Monosporic isolates of the *P. griseola* fungus can be obtained directly by placing the sample, whether a diseased leaf or pod, in a humid chamber. The purpose is to activate the fungal development and the production of conidia.

### Materials

– Humid chamber
– Petri dishes
– Sterilized distilled water
– Filter paper
– V-shaped glass rods
– Samples of diseased leaves or pods
– Needles
– Pasteur pipette
– Water agar or PDA culture medium
– Glass scraper
– Petri dishes containing V8 culture medium
– Stereoscope
– Bunsen burner
– Incubator

**Photo VI–5**


**Photo VI–6**

Photo VI–7

Photo VI–8

Photo VI–9

Photo VI–10

## Processing steps

- ***Step 1***
  Put filter paper in a petri dish and place on it some V-shaped glass rods. Then wet the paper with sterilized distilled water (**Photo VI–7**).

- ***Step 2***
  On the V-shaped rods, place diseased leaves with the lower surface uppermost, taking care to prevent contact between the diseased tissue and the wet paper. If diseased pods are used, place them so that the lesions face upwards (**Photos VI–8** and **VI–9**).

- ***Step 3***
  Cover the dish and store it for 2 or 3 days in a dark place at room temperature. After this time, examine the fungal development and synnemata (**Photo VI–10**), using a stereoscope.

- ***Step 4***
  When the synnemata have sporulated, take a small piece of agar or PDA culture medium with the point of a flame-sterilized needle and gently touch one or more sporulated synnemata so that conidia will adhere to the agar.

- ***Step 5***
  Draw up sterilized distilled water into a sterilized pipette and wash the conidia off the piece of agar. The agar will disintegrate in the washing (**Photo VI–11)**.

- ***Step 6***
  Then use a glass scraper to spread the conidial suspension obtained in *Step 5* across the culture medium, which may be either PDA medium or water agar (**Photo VI–12**).

- ***Step 7***
  Incubate the whole at 24 °C for 24 h, by which time the conidia will have germinated.

- ***Step 8***
  Examine the germinated conidia under the stereoscope (**Photo VI–13**). Select one that is very isolated from the others and remove it with a flame-sterilized needle, transferring it to a petri dish containing V8 culture medium. Incubate the dish at 24 °C.

  After 5 days, a grey spot should be seen at the site where the conidium was cultured onto the medium (**Photo VI–14**). This indicates that isolation was successful and that the fungus is in full development.

**Photo VI–11**

**Photo VI–12**

**Photo VI–13**

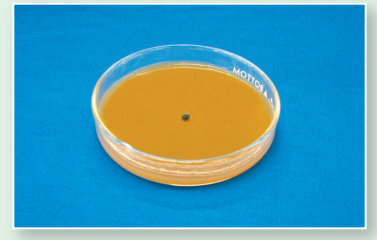


**Photo VI–14**

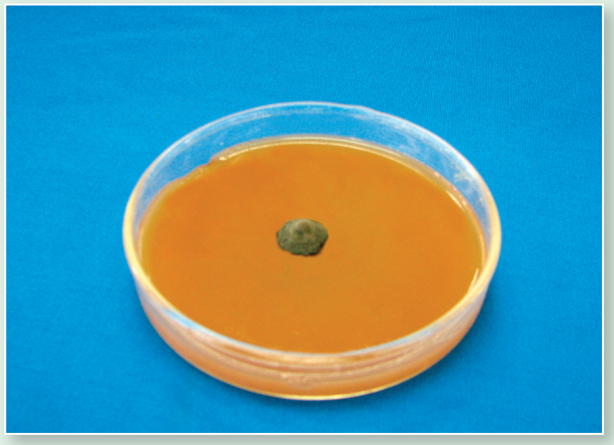


Photo VI–15

Photo VI–16

The fungus will continue growing and, 25 days later, its colony will have expanded (**Photo VI–15**), growing in a columnar fashion. The colony's diameter will be no larger than 1 cm after this period of time.

This monosporic isolate could be then used for diverse procedures such as inoculation tests, DNA extraction from mycelium, and conservation.

## D. Increasing *P. griseola*

Increasing must begin with a monosporic isolate (**Photo VI–15**).

### Processing steps

- ***Step 1***
  Select the monosporic isolate to be increased.

- ***Step 2***
  Prepare a conidial suspension by depositing about 10 drops of sterilized distilled water on the colony, using a pipette. Then scrape the surface of the colony with the tip of the pipette (or a sterilized spatula) to detach conidia from the conidiophores (**Photo VI–16**).

- ***Step 3***
  Use the same sterilized pipette to transfer the conidial suspension to a petri dish containing V8 culture medium. Spread the suspension, using a flame-sterilized glass scraper, over the medium's entire surface. Prepare several petri dishes using the same procedure.

- ***Step 4***
  Incubate the petri dishes at 24 °C for 12 days.

## E. Inoculating plants

### *1. Inoculum for use in the greenhouse*

#### Processing steps

- ***Step 1***
  After 12 days of incubation, select those petri dishes carrying the best growths.

- ***Step 2***
  Add sterilized distilled water to the selected fungal growths and then scrape the isolates' surfaces with a sterilized spatula to detach the conidia (**Photo VI–17)**.

- ***Step 3***
  Filter all the conidial suspensions through sterilized gauze to separate residues of the V8 culture medium. Collect the filtrate, containing conidia from all the petri dishes, in a medium-sized beaker (**Photo VI–18**).

**Photo VI–17**

**Photo VI–18**


**Photo VI–19**

**Photo VI–20**

The separation of solid particles will facilitate the conidial count and also prevent the nozzle of the DeVilbiss nebulizer from blocking. The nebulizer is used, together with a compressor, to apply inoculum to plants. An airbrush or liquid atomizer can be used instead of a DeVilbiss.

- ***Step 4***

  Use a microscope to count the conidia observed in the central grid of a hemocytometer comprising one central and four outer squares (**Photos VI–19** and **VI–20**). Multiply the number of conidia counted by 10,000. The concentration of inoculum should be, in this case, $2 \times 10^4$ conidia per milliliter. Given the final volume of inoculum to be sprayed and given the concentration of conidia in the original inoculum, use the following formula to find the volume of the original inoculum needed for spraying:

  $$V_1 \times C_1 = V_2 \times C_2$$

  For example, if 8 conidia are counted in the hemocytometer, then:

  $$8 \times 10{,}000 = 80{,}000$$
  $$= 8 \times 10^4 \text{ conidia per milliliter}$$

  This is the concentration of the original inoculum (i.e., the filtrate in *Step 3*). If 250 mL of inoculum with a concentration of $2 \times 10^4$ conidia per milliliter is needed

then, by substituting these data in the formula described above, the value of the volume of the original (also called *initial*) inoculum needed ($V_1$) can be found, that is:

$$V_1 \times 8 \times 10^4 = 500 \text{ mL} \times 2 \times 10^4$$

Thus,

$$V_1 = \frac{500 \text{ mL} \times 2 \times 10^4}{8 \times 10^4} = 125 \text{ mL}$$

Therefore, to obtain the final concentration desired, take 125 mL of initial inoculum and complete it to 500 mL with sterilized distilled water.

- ***Step 5***
  Once having obtained the concentration of conidia per milliliter of water required for inoculation, pour 250 mL of this inoculum suspension into a 250-mL erlenmeyer flask. Connect it to a DeVilbiss nebulizer or airbrush and this to a compressor.

- ***Step 6***
  Spray 17-day-old plants on their primary trifoliate leaf (**Photo VI–21**) to inoculate them. The volume of inoculum applied to the leaf is calculated so that the foliar surface is left entirely wet avoiding inoculum draining or running off.

**Photo VI–21**

- ***Step 7***
  Place the inoculated plants in a humid chamber set at a temperature between 22 and 24 °C and relative humidity at more than 90%. Leave the plants for 96 h. Then remove them from the chamber and leave them on greenhouse tables until evaluation.

- ***Step 8***
  Ten days after their removal from the chamber, examine the plants for symptoms of angular leaf spot (**Photo VI–22**).

  To evaluate them, use the **CIAT standard scale**, with scores ranging from 1 to 9, where:

  1 to 3 indicate a resistant plant
  4 to 6 indicate a plant with an intermediate reaction
  7 to 9 indicate a susceptible plant

**Photo VI–22**

## 2. *Inoculum for use in the field*

### Materials

- Petri dishes
- Growths of *P. griseola* on V8 culture medium
- Industrial blender
- Large receptacle
- Running water
- Funnel
- Hemocytometer
- Spatula
- Motorized back sprayer

## Processing steps

- ***Step 1***
  Pour running water into the bowl of an industrial blender. Use a spatula to add several dishes of fungal cultures and their V8 media (**Photo VI–23**). About 70 dishes are needed for a homogeneous product of 20 L.

- ***Step 2***
  Blend the bowl's contents until a homogeneous product is obtained.

- ***Step 3***
  Quantify the conidial concentration in the homogenized inoculum, using a hemocytometer. Then prepare the final solution of inoculum according to the following information:

  The amount of artificial inoculum needed for 1 hectare of a bean crop in the field is at least 70 petri dishes of abundantly sporulating growths of *P. griseola* diluted in 140 L of water at the required concentration $2 \times 10^4$ conidia/mL.

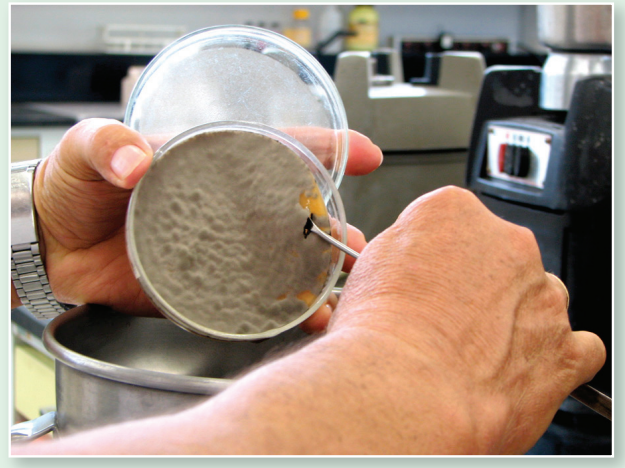


**Photo VI–23**


Photo VI–24

- ***Step 4***
  Pour the inoculum into an easily manageable receptacle, using a funnel (**Photo VI–24**). Firmly close the receptacle and constantly check for fissures or leaks.

  Always carry out inoculations with this pathogen in the early evening, when temperatures are dropping and relative humidity is increasing. Use a motorized back sprayer to inoculate the crop.

## F. Conserving the fungus for storage

The *P. griseola* fungus can be conserved by lyophilization, on pieces of filter paper kept in glassine envelopes, or on squares of filter paper impregnated with the fungus suspended in peptone-sucrose solution and kept in glassine envelopes at -20 °C.

### *1. Lyophilization*

#### Materials

- Tree manifold lyophilizer with two types of support (rack and tree) and 48 ports for vials
- 0.5-mL neutral-glass vials for lyophilizing
- Fungal growth on V8 culture medium
- Long Pasteur pipettes
- Filter paper
- Marker pen and notebook

- Scissors
- Peptone at 10%
- Freezer
- Sucrose at 20%
- Tweezers or inoculation needle
- Distilled water
- Propane gas torch
- Bunsen burner
- Cotton wool
- Autoclave
- Large receptacle

***Note:*** Fungal growth must be prepared 12 days before lyophilization (**see *VI.D.* above**). The fungus should have sporulated and be free of contaminants.

## Processing steps

- ***Step 1***
  Write in either small lettering or code on 1- to 2-cm squares of filter paper to identify the isolates to be lyophilized. Include the fungus's genus and species names, isolate name (i.e., the code the laboratory uses), and storage date.

  If you write in code, write the full information in a notebook.

Photo VI–25

Photo VI–26

Photo VI–27

- ***Step 2***
  Use sterilized scissors to cut out the pieces of filter paper identifying the isolates and introduce each piece to the bottom of a vial.

- ***Step 3***
  Then prepare a cotton wool plug to stopper each vial so that it plugs the top two-thirds, as follows:

  – Tease out a piece of cotton wool until it measures about 5 x 3 cm and roll it around a tweezers arm or inoculation needle, pressing between fingers and thumb until a plug is formed (**Photos VI–25**, **VI–26**, and **VI–27**). Leave a piece of unrolled cotton wool on the end opposite the plug.
  – Introduce the plug into the vial and gently withdraw the tweezers arm or needle with one hand while holding the plug in the vial with the other.
  – Prepare additional vials with their respective plugs to have spares on hand as needed. If a plug disintegrates on handling, it can therefore be replaced by one from the additional vials.

- ***Step 4***
  Autoclave all the vials with their respective plugs for 40 min until sterilized.

- ***Step 5***
  **Preparing peptone and sucrose solutions.**
  Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Then prepare a 20% sucrose solution by dissolving 20 g of sucrose in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix the two solutions in equal parts in a sterilized receptacle to make a homogeneous peptone-sucrose solution.

- ***Step 6***
  Use a long Pasteur pipette to draw up 2 to 3 mL of the peptone-sucrose solution and deposit them on a fungal culture (**Photo VI–28**). Homogenize the fungus (i.e., its mycelium and conidia) within the solution by first scraping the culture with the pipette and then by repeatedly drawing up and expelling the mixture, using the same pipette over the same medium. If the pipette is not accidentally contaminated with non-sterile surface it can be used throughout the entire process

**Photo VI–28**

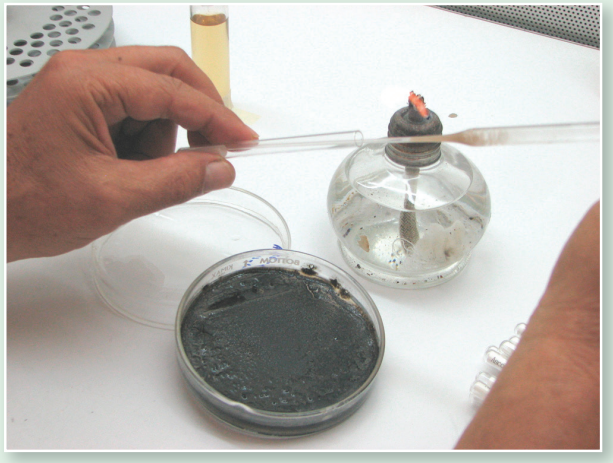


Photo VI–29

Photo VI–30

- ***Step 7***

  Again use the same Pasteur pipette to draw up 2 to 3 mL of the homogenized suspension (**Photo VI–29**) and deposit them inside a vial, as follows:

  – In one hand take the Pasteur pipette and, in the other, the vial with its respective plug, as prepared in *Step 3* above.
  – Hold the pipette between the index finger and thumb, and use the little finger (or pinky) of the same hand to hold the loose end of the cotton wool plug against the palm. Carefully withdraw the plug from the vial, keeping it firmly against the palm. Then deposit the fungal suspension in the bottom of the vial, which should have the filter paper identifying the isolate.
  – Stopper the vial by gently introducing the plug (**Photo VI–30**), which had been held between the little finger and palm, back into the vial.

  If the cotton wool plug comes into contact with another surface or some substance it becomes contaminated. Discard it therefore and replace it by one of the additional plugs that had been prepared in *Step 3* above for such an eventuality.

- ***Step 8***
  Use flame-sterilized scissors to cut off that part of the plug remaining outside the vial (**Photo VI–31**). Finally, introduce the rest of the plug, using the point of a scissors arm, until 1 cm is left between the plug and the lip of the vial (**Photo VI–32**).

- ***Step 9***
  Place all the vials in a rack. Then push the plugs to the bottom of each vial until it touches the piece of filter paper identifying the isolate. Use a long object such as an inoculation needle, constantly flame-sterilizing it to prevent contaminating the plugs (**Photo VI–33**).

  The upper part of the vial, which has no plug, is sealed after lyophilization (see *Step 14* below).

- ***Step 10***
  Once the vials are organized, freeze them at 0 °C for 15 min. When the samples are frozen, lyophilization can begin.

**Photo VI–31**

**Photo VI–32**

**Photo VI–33**

Photo VI–34

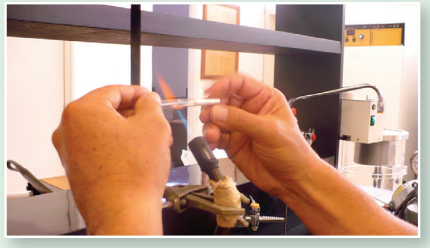


Photo VI–35

Photo VI–36

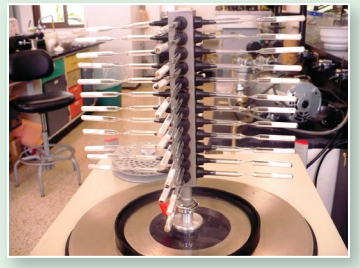


Photo VI–37

- ***Step 11***
  Remove the rack from the freezing process and place it inside the lyophilizer dome. Switch on the machine (**Photo VI–34**). The temperature inside the apparatus will drop to -55 °C, at which point a vacuum pump will begin extracting water, drying the samples. This process will take 20 to 22 h to complete.

- ***Step 12***
  The next day, switch off the lyophilizer and remove the rack of vials. Prepare a propane gas torch for constricting the vials at the end opposite the sample. That is, make a neck in each vial by softening the glass with the torch's flame and pulling gently on the ends of the vial. (**Photos VI–35** and **VI–36**).

  The procedure requires considerable care to prevent burns to the hands. This step reduces the space by which air and therefore humidity can reach the sample before sealing is completed.

- ***Step 13***
  Once all the vials have been constricted, place them on the ports of the lyophilizer tree, inserting the open end into each port. Thus, the end carrying the sample remains visible (**Photo VI–37**). Repeat the drying process in the lyophilizer for about 1 h to eliminate any humidity that may have reached the sample during *Step 12*.

- ***Step 14***
  Without switching off the lyophilizer, seal the vials, one by one, by vacuuming and then using the propane gas torch to melt the glass and thus constrict the neck (**Photo VI–38**). Once the vials are sealed, lyophilization is completed.

***Note:*** If vacuum is lost on sealing a vial, remove this part of the vial and replace it with a new one. Wait for the vacuum to reach the point of sealing before continuing to seal the other vials.

How long a lyophilized sample can last in storage has not been formally established. However, in our laboratory, lyophilized samples have been recovered after 30 years of storage. Lyophilized samples do have one disadvantage: to recover a sample, the entire vial must be destroyed. Several copies of each lyophilized sample must therefore be made to maintain an adequate collection.

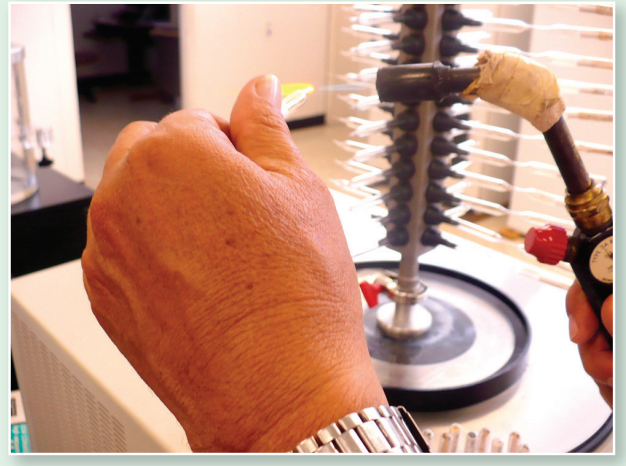
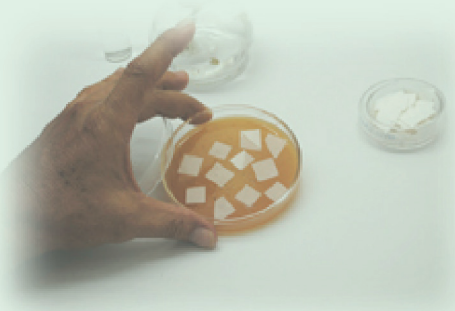


**Photo VI–38**



## 2. Conservation as fungal growth on filter paper at 20 °C

This method permits long-term conservation of microorganisms.

### Materials

- Petri dishes containing V8 culture medium
- Sterilized filter paper
- Growths of *P. griseola*
- Tweezers
- Sterilized petri dishes
- Sterilized distilled water
- Pipette or spatula
- Glassine envelopes
- Autoclave
- Scissors
- Incubator
- Marker pen

### Processing steps

- ***Step 1***
  Cut the filter paper into 60 to 80 1-cm squares, place them in a petri dish or lidded container, and sterilize by autoclaving.

- ***Step 2***
  Place 10 of the squares in each petri dish containing V8 culture medium.

- ***Step 3***
  Prepare a suspension of conidia and mycelium from a fungal isolate, as follows:

  – Deposit sterilized distilled water on an isolate already grown in a petri dish.
  – Scrape the surface of the isolate with the tip of a pipette or spatula until a suspension is generated.

- ***Step 4***
  Draw up a little of this suspension with the same pipette and deposit one drop on each square placed on V8 medium.

- ***Step 5***
  Incubate the dishes at 24 °C for 12 days.

- ***Step 6***
  After 12 days, use sterilized tweezers to remove the squares of filter paper from the petri dishes and, complying with all conditions for asepsis and sterilization, deposit them in empty sterilized petri dishes. Place the squares so that the fungus faces downwards. This should ensure that the papers will not curl on drying. Dry the squares at 24 °C for 7 days.

- ***Step 7***
  Use sterilized tweezers to remove the squares and transfer them to sterilized glassine envelopes. Introduce these into a larger glassine bag for storage (**Photos VI–42** and **VI–43**). Write information on the *P. griseola* fungus on this last big bag and store at -20 °C.

## 3. Conservation as a suspension on filter paper at -20 °C

### Materials

- Peptone-sucrose solution, prepared as described in ***VI.F.1, Step 4***
- Petri dishes containing sporulating fungal growths
- Sterilized petri dishes
- Sterilized filter paper
- Tweezers
- Pipettes or spatula
- Glassine envelopes
- Marker pen

## Processing steps

- ***Step 1***
  Take a petri dish containing a sporulated fungal isolate and add 2 mL of peptone-sucrose solution (**Photo VI–39**).

- ***Step 2***
  Use a sterilized spatula (or the same pipette used to add the solution) to scrape the surface of the isolate thus detaching conidia (**Photo VI–40**) and obtaining a conidial suspension.

- ***Step 3***
  Use sterilized tweezers to place twenty 0.5-cm squares of filter paper in a petri dish and impregnate them with the fungus suspended in the peptone-sucrose solution. (**Photo VI–41**).

- ***Step 4***
  Use sterilized tweezers to remove the squares and place them on filter paper lining the bottom of another, sterilized petri dish. Dry at 24 °C for 7 days.

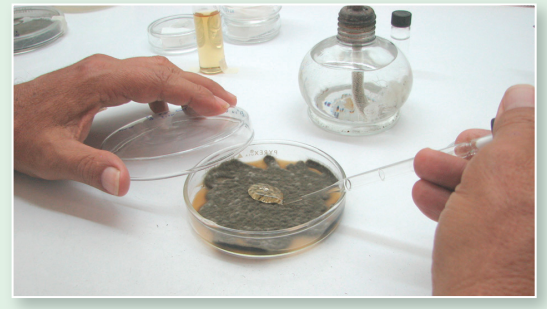


**Photo VI–39**

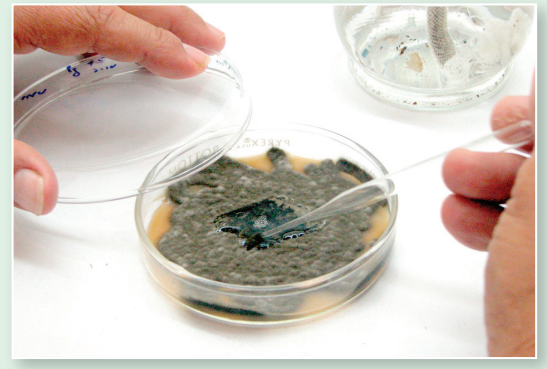


**Photo VI–40**

**Photo VI–41**

Photo VI–42

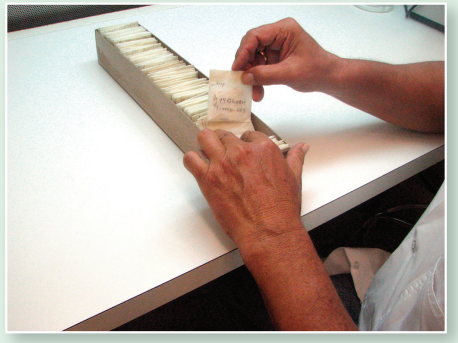


Photo VI–43

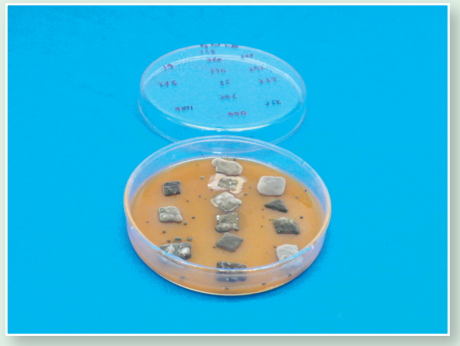


Photo VI–44

- ***Step 5***
  After drying, place the squares in sterilized glassine envelopes (**Photo VI–42**) for storage. Write on the envelopes information on the fungus such as storage date and strain name (**Photo VI–43**). Store at -20 °C.

## G. Proof of storage

The purpose of this test is to guarantee that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.

### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with fungal suspension and then dried for 7 days at 24 °C.

- ***Step 2***
  Culture the square on V8 medium. After 5 days, the pathogen should be growing on the medium. (**Photo VI–44**).

- ***Step 3***
  Examine the fungal growth under the stereoscope.
  If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

## H. Recovering stored fungus

### Materials (Photo VI–45)

- Vial containing the lyophilized fungus
- Peptone-sucrose solution, prepared as described in ***VI.F.1, Step 4***
- Petri dishes containing V8 culture medium
- Bulb pipette
- Pestle
- Sterilized cloth napkin
- Tweezers
- Incubator

Photo VI–45

Photo VI–46

Photo VI–47

Photo VI–48

Photo VI–49

## 1. Conserved by lyophilization

### Processing steps

- ***Step 1***
  Select a vial containing the lyophilized fungus to be restored. Break the vial by tapping it with a pestle (**Photo VI–46**) on the end opposite that carrying the fungus. Use the cloth napkin to cushion the blows and contain the breaking glass. Use tweezers to remove the cotton wool plug from inside the vial (**Photo VI–47**).

- ***Step 2***
  With a pipette, deposit 4 drops of peptone-sucrose solution in the open half of the vial. The lyophilized conidia will then become suspended in the solution and so can be removed from the vial (**Photo VI–48**).

- ***Step 3***
  Culture the conidial suspension into a petri dish containing the V8 medium to reinitiate fungal growth (**Photo VI–49**).

## 2. *Conserved as fungal growth on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***VI.F.2, Step 6***) containing squares of filter paper carrying the fungal isolate to be restored. Use sterilized tweezers to select a square and transfer it to a petri dish (**Photo VI–50**) containing V8 culture medium.

- ***Step 2***
  Use a pipette to deposit 1 or 2 drops of peptone-sucrose solution on each square, thus hydrating the fungus. Then use the tip of the same pipette to spread the isolate over the entire surface of the medium (**Photo VI–51**). This will increase the area over which the fungus will grow.

- ***Step 3***
  Incubate the petri dishes at 24 °C. Fungal growth will reactivate and, after 12 days, the fungus will be ready for use in later procedures.


**Photo VI–50**

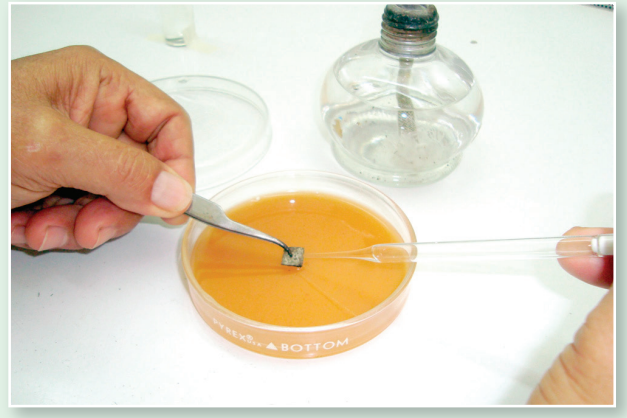



**Photo VI–51**

## *3. Conserved as a suspension on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***VI.F.3, Step 5***) containing squares of filter paper carrying the fungal isolate to be restored. Remove 1 or 2 squares and transfer them to petri dishes containing V8 culture medium.

- ***Step 2***
  Incubate the petri dishes at 24 °C. After 2 or 3 days, fungal growth should be seen. Twelve days after incubation, the fungus will be ready for increasing or other uses.

# Practical Guide 7

*Rhizoctonia solani*

(Rhizoctonia root rot)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
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## Contents

# Considerations

Photo VII–1

Photo VII–2

Root rot caused by *Rhizoctonia solani* Kuhn is one of the most common root rot affecting beans. The fungus develops when temperatures are moderate to low—the ideal being 18 °C—and soil humidity is moderate to high.

The symptoms, known as cankers, are reddish-brown concave lesions that appear on stems (**Photo VII–1**) and on the main root. The pathogen can eventually cause lodging (**Photo VII–2**), a condition also known as *damping off*. Susceptible plants suffer serious damage in the first 2 weeks after planting.

# Procedures

## A. Collecting and shipping samples

To isolate the *Rhizoctonia solani* fungus, first collect root tissues from bean plants presenting typical symptoms of the disease. Use a spatula or tongue depressor to dig up a diseased plant as completely as possible. Use running water to wash the soil off the roots. Place the plant on paper towels and leave until the roots are dry.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying samples
- Marker pen and fieldbook

- ***Paper towels.*** Wrap samples collected from either the roots or stems of diseased bean plants in paper towels that will absorb their moisture. If no towels are available, use similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and will therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the development of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach labels to their corresponding samples.

## *Recommendations*

– Indicate on the label if collection was in a farmer's field or on an experiment station.
– If there are not enough labels for all the samples, code the bags and record their corresponding information in a fieldbook.
– Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once in the laboratory, and if they are not to be processed immediately, leave them out to finish drying on paper towels spread on a bench.

### Processing steps

- ***Step 1***
  Take samples from roots presenting rhizoctonia root rot.

- ***Step 2***
  Wrap each sample in a paper towel, place it in a paper bag or envelope, and firmly attach the corresponding label.

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons explained above.

## B. Preparing PDA culture medium

The letters in the abbreviation "PDA" correspond to components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- PDA powder (39 g)
- 1 L distilled water
- Two 1000-mL erlenmeyer flasks
- Balance
- Large receptacle (e.g., beaker)
- Petri dishes
- Autoclave

Photo VII–3

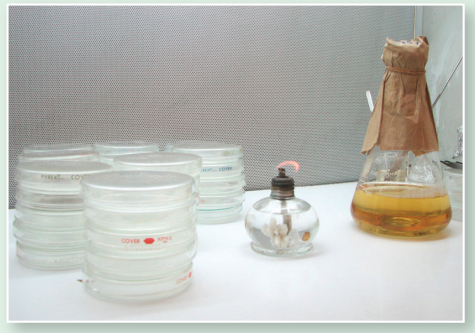

Photo VII–4

Photo VII–5

### Preparation

- Weigh the ingredients (**Photo VII–3**) and place them in a large receptacle such as a beaker. Add 1 L of distilled water. Pour the resulting solution (PDA culture medium) into two erlenmeyer flasks at 500 mL each.
- Sterilize the flasks containing the medium by autoclaving for 40 min at 121 °C and a pressure of 20 psi.
- Leave the sterilized medium until cool enough (**Photo VII–4**) to pour into petri dishes at 25 mL per dish (**Photo VII–5**).

## C. Isolating *R. solani*

### *1. On PDA culture medium*

The protocol for isolating *R. solani* is simpler compared to other fungi because of its fast growth. Two days after culturing the pathogen on PDA medium, the fungus can be seen growing on the medium. After several days, the isolate will become brown-colored and will completely cover the medium in the petri dish.

### Materials

- Laminar-flow chamber
- Paper towels
- Scissors
- Incubator

- 2.5% sodium hypochlorite
- Tweezers
- 60- and 100-mm petri dishes
- Petri dishes containing PDA culture medium
- Sterilized distilled water
- Samples of diseased tissue

***Note:*** All procedures must be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

## Processing steps

- ***Step 1***
  Use sterilized scissors to cut diseased tissue into several pieces (**Photo VII–6**).

- ***Step 2***
  Place the sample pieces in a 60-mm petri dish containing a solution of 2.5% sodium hypochlorite. Leave for 3 min to disinfect (**Photo VII–7**). Then rinse the disinfected pieces three times with sterilized distilled water.

- ***Step 3***
  Place the disinfected and washed pieces on sterilized paper towels to dry for 10 min (**Photo VII–8**).

**Photo VII–6**

**Photo VII–7**

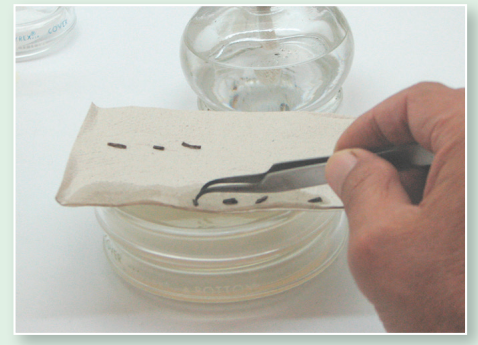

**Photo VII–8**

Photo VII–9

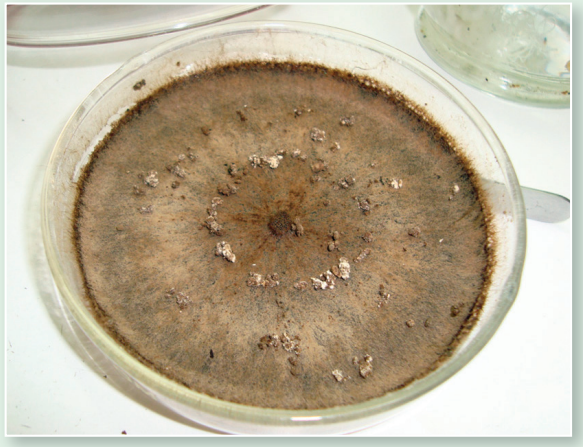

Photo VII–10

- ***Step 4***
  Once the pieces are dry, use sterilized tweezers to select 3 pieces and culture them in a petri dish containing PDA medium. Repeat the procedure for all the samples. (**Photo VII–9**).

- ***Step 5***
  Incubate all the dishes at 24 °C for 10 days, by which time the fungus should be producing mycelium and sclerotia. The latter will appear as brown-colored spots (**Photo VII–10**). Ten days later, the isolate should be ready for use, and can also be purified as a monomycelial isolate.

***Note:*** The fungal culture is off-white in color during its early stages of growth.

## 2. As a monomycelial isolate

Because a sample of diseased plant tissue may carry a mixture of pathogenic strains, the fungus needs to be purified. A genetically pure strain of *R. solani* can be obtained through monomycelial isolation, using hyphal tips. This procedure is used for fungi that do not produce structures like spores or conidia.

### Processing steps

- ***Step 1***
  Remove a 0.5-cm square of PDA culture medium on which the fungus is growing (see *Step 5* above) and transfer the square to the center of a petri dish containing fresh PDA medium.

- ***Step 2***
  Incubate at 24 °C for 24 h. The next day, examine the fungus under the stereoscope in a laminar-flow chamber for mycelial growth. Focus on the edges of the colony to identify growing hyphal tips.

- ***Step 3***
  Once hyphal tips are identified, use a flame-sterilized needle to cut out a piece of agar carrying a single tip of one of a hyphy, as follows:

  – Take care not to touch other hyphae surrounding the selected tip.

- Transfer the piece of agar carrying the hyphal tip to another petri dish containing fresh PDA culture medium. Continue with other dishes, placing only one hyphal tip per dish.
- Incubate all the dishes at 24 °C for 10 days. By this time, a culture should be obtained that is similar to the one described in *Step 5* above, except that it is genetically pure. The fungus can then be increased for multiple uses as storage, or inoculation either in the greenhouse or field

Photo VII–11

## D. Increasing *R. solani*

Increasing the fungus *R. solani* for the greenhouse by using a mixture of either soil with sand and chopped potato, or of soil and bean flour.

### *1. On a soil, sand, and potato mixture*

#### Materials

- Petri dishes containing monomycelial isolates of *R. solani*
- Sterilized soil
- Tray
- Sand
- Autoclave
- 200 g potato and sharp knife
- 1-cm-diameter punch
- 1000-mL erlenmeyer flask
- Incubator

## Processing steps

- ***Step 1***
  In a tray, mix 4 parts of soil to 1 part of sand.

- ***Step 2***
  Chop the potatoes into small pieces (**Photo VII–11**) and combine them with the soil mixture at a rate of 100 g of chopped potato to 1600 g of soil-sand mixture. (**Photo VII–12**).

- ***Step 3***
  Introduce 400 mL of this new mixture into a 1000-mL erlenmeyer flask (**Photo VII–13**). Sterilize the mixture two times by autoclaving.

- ***Step 4***
  Take a petri dish carrying a monomycelial culture of *R. solani* (**see *VII.C.2* above**) and extract 10 discs of the medium on which the fungus was grown, using a 1 cm-diameter punch. Carry out the entire procedure under aseptic and sterilized laboratory conditions.

- ***Step 5***
  Add the 10 discs to the erlenmeyer flask (see *Step 3* above) (**Photo VII–14**). Manually shake the flask until the discs are distributed throughout the entire mixture of soil, sand, and chopped potato.

Photo VII–12


Photo VII–13


Photo VII–14

Photo VII–15

- ***Step 6***
  Incubate the flask at 24 °C for 12 days. By this time, the fungus should have colonized the soil-sand and potato mixture, and be ready for inoculation in greenhouse tests.

## 2. On a soil and bean flour mixture

### Materials

- Two petri dishes containing a monomycelial isolate of *R. solani*
- 1 kg sterilized soil
- Aluminum foil
- 10 g bean flour
- Wrap paper
- 50 mL distilled water
- Autoclave
- 1-cm-diameter punch
- Tray or other receptacle

### Processing steps

- ***Step 1***
  In a receptacle that can be autoclaved such as a tray, deposit 1 kg of soil and add 10 g of bean flour (**Photo VII–15**). Homogenize the mixture and distribute it at no deeper than 3 cm.

- ***Step 2***
  Add 50 mL of distilled water to this mixture to humidify the soil.

- ***Step 3***
  Cover the receptacle with aluminum foil and then again with wrapping paper. Autoclave the whole (**Photos VII–16** and **VII–17**).

- ***Step 4***
  After autoclaving, leave the tray of soil to cool. Then remove the covers and add 1 cm-diameter discs of monomycelial isolate (**Photo VII–18**) at 25 discs for every kilogram of soil (**Photo VII–19**).

- ***Step 5***
  Re-cover the tray with the same foil and paper used for autoclaving. Leave at room temperature (22 °C) for 15 days, by which time the discs will have produced mycelium and colonized the soil (**Photo VII–20**).

**Photo VII–16**

**Photo VII–17**

**Photo VII–18**

**Photo VII–19**

**Photo VII–20**

Photo VII–21

Photo VII–22

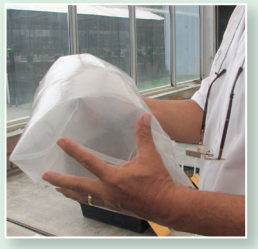

Photo VII–23

Photo VII–24

Photo VII–25

# E. Inoculating plants in the greenhouse

## *1. Using inoculum multiplied on a soil, sand, and potato mixture*

### Processing steps

- ***Step 1***
  Add, at a 2% rate, inoculum that was multiplied on a soil, sand, and potato mixture to soil that will be used for inoculation (**Photos VII–21** and **VII–22**).

- ***Step 2***
  Place the soil and inoculum in a plastic bag and shake it several times to ensure as homogeneous a mix as possible (**Photo VII–23**).

- ***Step 3***
  Fill trays or pots with planting soil and make rows in it. Within the rows sow the seeds of the bean genotypes being inoculated (**Photo VII–24**). Cover them with 100 g of the soil-inoculum mixture (**Photo VII–25**). Water the whole with running water. To scale up this experiment trays with bigger capacity could be used. It is recommended that containers for this inoculation testing should not be less than 20 cm deep to avoid over exposure of the root system to the inoculum which will kill the plants.

- ***Step 4***
  Evaluate the planted genotypes 14 days after inoculation, using the **CIAT standard scale**, with scores ranging from 1 to 9, where:

  1 to 3 indicate a resistant plant
  4 to 6 indicate a plant with an intermediate reaction
  7 to 9 indicate a susceptible plant

- ***Step 5***
  Also evaluate the incidence of disease in each genotype. To do this, examine the ratio of diseased plants, regardless of severity level, to the total number of seeds sown. Do not include those seeds that did not germinate for reasons other than the Rhizoctonia infection.

## 2. *Using inoculum multiplied on a soil and bean flour mixture*

### Processing steps

- ***Step 1***
  Add, at a 2% rate, the inoculum that was multiplied on a soil and bean flour mixture to the planting soil to be inoculated. If the inoculum was prepared on 1 kg of soil and bean flour mixture, then mix this with 50 kg of planting soil (**Photo VII–26**).

**Photo VII–26**

Photo VII–27

Photo VII–28

Photo VII–29

- ***Step 2***
  If the volumes to be mixed are large, use a motorized mixer (**Photo VII–27**).

- ***Step 3***
  Fill 2-kg pots of the mixture and plant the seeds (**Photo VII–28**). A mixture of 1 kg of soil-bean flour and inoculum will be sufficient for 25 2-kg pots.

- ***Step 4***
  Evaluate the bean genotypes 14 days after inoculation (**Photo VII–29**), using the **CIAT standard scale**, described above in *Step 4*.

## F. Conserving the fungus for storage

The fungus *R. solani* can be conserved either on filter paper at -20 °C or as a suspension in peptone-sucrose solution.

### *1. Conservation as fungal growth on filter paper at -20 °C*

This method permits long-term conservation of microorganisms.

## Materials

- Petri dishes containing PDA culture medium
- *R. solani* grown on PDA culture medium
- Sterilized filter paper
- Incubator
- Tweezers
- Autoclave
- Sterilized petri dishes
- Glassine envelopes
- Sterilized distilled water
- Marker pen
- Pipette

## Processing steps

- ***Step 1***
  Cut the sterilized filter paper into 60 to 80 1-cm squares, place them in a petri dish or other lidded container, and sterilize them by autoclaving.

- ***Step 2***
  In each petri dish, place 10 squares of sterilized filter paper on PDA culture medium.

- ***Step 3***
  Prepare a suspension of mycelium from a fungal isolate, as follows:

  – Add sterilized distilled water to a petri dish containing fungus grown on PDA culture medium.
  – Scrape the surface of with the fungal grow with the tip of a pipette to form a suspension.
  – Draw up some of this suspension in the same pipette and place one drop on each of the squares of sterilized filter paper.

- ***Step 4***
  Incubate the dishes at 24 °C for 12 days.

- ***Step 5***
  After incubation, use sterilized tweezers to remove the squares of filter paper, together with the fungus growing on them, from the medium. Follow all conditions for asepsis and sterilization and place each square in an empty sterilized petri dish so that the fungus faces downwards. This should prevent the papers from curling on drying. Dry the squares at 24 °C for 7 days.

- ***Step 6***
  Use sterilized tweezers to transfer the dried squares to sterilized glassine envelopes. These, in their turn, are introduced into another larger glassine bag. On each envelope write all information on the fungus inside such as storage date and strain name. Store the *R. solani* fungus at -20 °C.

## 2. *Conservation as a suspension on filter paper at -20 °C*

### Materials

- Peptone at 10%
- Distilled water
- Sucrose at 20%
- Autoclave
- Petri dishes containing 10-day-old fungus
- Receptacle
- Spatula
- Tweezers
- Filter paper
- Glassine envelopes
- Petri dishes
- Marker pen

### Preparing the peptone and sucrose solutions

Prepare the two solutions separately. Dissolve 10 g of peptone in 100 mL of distilled water, thus making a peptone solution at 10%. Also prepare a sucrose solution at 20% by dissolving 20 g of sucrose in 100 mL of distilled water. Autoclave the two solutions until sterilized. Then mix equal parts of each solution in a sterilized receptacle to make a homogeneous peptone-sucrose solution.

Photo VII–30

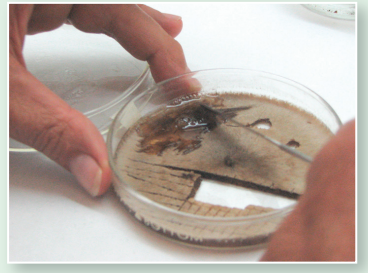

Photo VII–31

Photo VII–32

Photo VII–33

## Processing steps

- ***Step 1***
  Take a petri dish containing purified and increased fungus (**see *VII.C.2.* above**). Add 2 mL of the peptone-sucrose solution (**Photo VII–30**).

- ***Step 2***
  Use a sterilized spatula to scrape the surface of the fungus (**Photo VII–31**) to detach mycelium and sclerotia, and thus obtain a suspension.

- ***Step 3***
  Then use tweezers to select squares of filter paper (see above) and place them in the fungal suspension. Ensure that they are well impregnated (**Photo VII–32**).

- ***Step 4***
  Remove the squares of filter paper and place them on filter paper lining the bottom of another, sterilized petri dish. Dry the squares at 24 °C for 7 days (**Photo VII–33**).

- ***Step 5***
  After this time, place the squares in sterilized glassine envelopes (**Photo VII–34**) for storage. Write on the envelopes information on the fungus such as storage date and strain name (**Photo VII–35**). Store at -20 °C.

## G. Proof of storage

The purpose of this test is to guarantee that the fungus, after having been dried for 7 days, is free of contaminants, is viable, and can be stored.

### Processing steps

- ***Step 1***
  Select a square of filter paper that had been impregnated with *R. solani* suspension and dried at 24 °C for 7 days (see *Step 4* above).

- ***Step 2***
  Culture the square on fresh PDA medium. After 5 days, the stored pathogen should be growing on the medium.

- ***Step 3***
  Examine the fungal growth under the stereoscope. If contaminating agents appear such as other fungi or bacteria, then discard both the sample checked and the other squares. Repeat the entire conservation procedure.

**Photo VII–34**

**Photo VII–35**

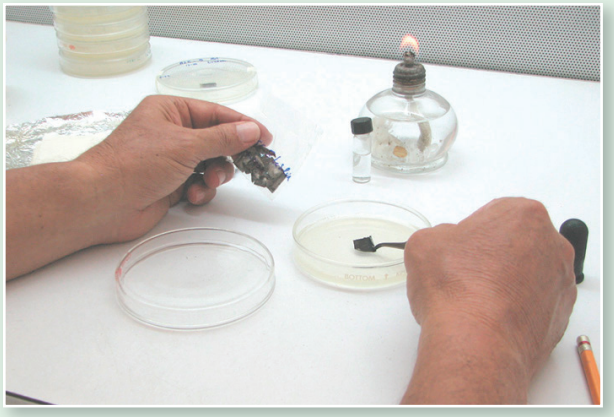

Photo VII–36

## H. Recovering stored fungus

### *1. Conserved as fungal growth on filter paper at -20 °C*

#### Processing steps

- ***Step 1***
  Select a glassine envelope (***VII.F.1., Step 6***) containing squares of filter paper carrying the fungal isolate to be restored. Use sterilized tweezers to select a square and transfer it to a petri dish containing PDA culture medium (**Photo VII–36**).

- ***Step 2***
  Incubate at 24 °C for 3 days, by which time the fungus will have reinitiated growth. After 8 days, it should be ready for multiplication.

## *2. Conserved as a suspension on filter paper at -20 °C*

### Processing steps

- ***Step 1***
  Select a glassine envelope (***VII.F.2., Step 5***) containing squares of filter paper carrying the fungal isolate to be restored. Remove 1 or 2 squares and transfer them to a petri dish containing PDA culture medium (**Photo VII–37**). Repeat the same procedure with other isolates.

- ***Step 2***
  Incubate the petri dishes at 24 °C for 12 days. After 3 or 4 days, check if a fungal growth has covered the medium in the petri dish. After 12 days, the fungus should be ready for new tests.

**Photo VII–37**

# Practical Guide 8

*Sclerotium rolfsii*

(Southern blight, southern stem rot)

# Managing the fungus in the laboratory

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Bean Plant Pathology

CIAT
International Center for Tropical Agriculture
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## Contents

# Considerations

**Photo VIII–1**

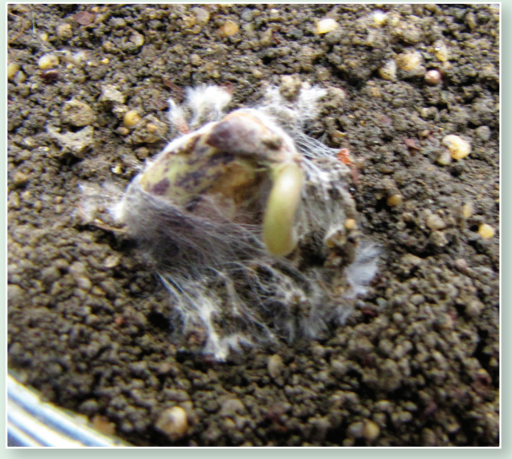

**Photo VIII–2**

The fungus *Sclerotium rolfsii* Sacc. causes the disease known as southern blight, southern stem rot.

It lives in soils across the tropics and subtropics, and appears particularly during rainy seasons when temperatures are high.

Characteristic symptoms of the disease first appear in the root neck of the bean plantlet, spreading through the stem. If attack is severe, white mycelium can be seen on the root neck, with sclerotia forming (**Photo VIII–1**). Plantlet hardly recover after these symptoms. **Photo VIII–2** shows a bean seed that has been attacked by the fungus during germination, and covered by the fungal mycelium. The sclerotia of *S. rolfsii* are spherical in shape, their color varying from white, as they start developing, to chestnut-colored as they mature over the next days.

# Procedures

## A. Collecting and shipping samples

To isolate the *Sclerotium rolfsii* fungus, first collect samples of the root neck from bean plants presenting symptoms of southern blight.

### Materials

- Paper towels
- Paper bags or envelopes
- Labels for identifying samples
- Marker pen and fieldbook

- ***Paper towels.*** Wrap samples of diseased bean tissue, usually collected from roots, in paper towels that will absorb their moisture. If no towels are available, use other similar materials such as paper napkins, toilet paper, facial tissues, and, as a last resort, newspaper.

- ***Paper bags or envelopes.*** Place the samples of diseased tissue, wrapped in absorbent paper, in paper bags or envelopes. Do not wrap in aluminum foil or use plastic bags, as they are not porous and would therefore contribute to the accumulation of humidity in the bags or wrappings. This would foster the development of saprophytic microorganisms, thus complicating the isolation of the targeted pathogen.

- ***Labels for identifying samples.*** Clearly identify each sample with the following information: bean variety or genotype; seed size and color; name of sampling place (i.e., site, province, department or state, and country); collection date; name of collector; and, where possible, the approximate latitude and longitude of the collection site. Firmly attach labels to their corresponding samples.

## *Recommendations*

- Indicate on the label if collection was in a farmer's field or on an experimental station.
- If there are not enough labels for all the samples, code the bags and record the full information in a fieldbook.
- Do not collect wet plant materials. If this is unavoidable before shipping, dry the samples with paper towels. Once in the laboratory, and if they are not to be processed immediately, leave them out to finish drying on paper towels spread on a bench.

### Processing steps

- ***Step 1***
  Take samples of roots from plants presenting southern blight.

- ***Step 2***
  Wrap each sample in a paper towel, place it in a paper bag or envelope, and firmly attach the corresponding label.

- ***Step 3***
  Send the samples as soon as possible to the site or laboratory where the fungus will be isolated. Never send samples in plastic bags or wrapped in aluminum foil for the reasons explained above.

## B. Preparing PDA culture medium

The letters of the abbreviation "PDA" correspond to components of the medium used for isolating the pathogen: potato, dextrose, and agar.

### Materials

- PDA powder, (39 g)
- 1 L distilled water
- Two 100-mL erlenmeyer flasks
- Balance
- Large receptacle (e.g., beaker)
- Autoclave
- Petri dishes

Photo VIII–3

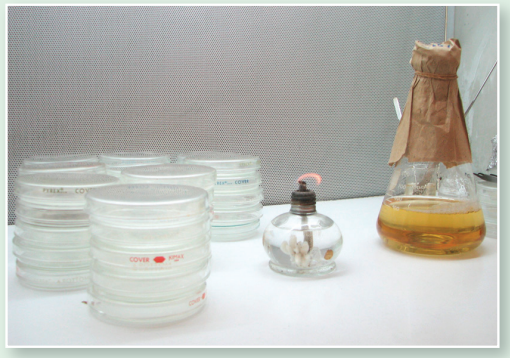

Photo VIII–4

Photo VIII–5

### Preparation

- Weigh the ingredients (**Photo VIII–3**), place them into a large receptacle such as a beaker, and add distilled water. Pour the resulting solution (PDA culture medium) into two erlenmeyer flasks at 500 mL each.
- Sterilize the flasks containing the medium by autoclaving for 40 min at 121 °C and a pressure of 20 lb.
- Leave the sterilized medium until cool enough (**Photo VIII–4**) to pour into petri dishes at 25 mL each (**Photo VIII–5**).

## C. Isolating *S. rolfsii*

### Materials

The fungus can be isolated by culturing on PDA medium which in turn is used to generate genetically pure cultures prepared by monohyphal purification. The materials needed for isolation include:

- Growths of fungus in petri dishes containing PDA culture medium
- Samples of diseased tissues
- Incubator
- Scissors
- Petri dishes containing PDA medium
- 60- and 100-mm petri dishes
- Tweezers
- Sterilized distilled water
- 0.5-cm-diameter punch
- 2.5% sodium hypochlorite

- Stereoscope
- Paper towels
- Needle
- Bunsen burner
- Glassine envelopes or glass tubes with seals
- Marker pen
- Laminar-flow chamber

***Note:*** All procedures must be carried out in a laminar-flow chamber, and all conditions of asepsis and sterilization as required for a laboratory must be met. In other words, best microbiological practices should always be applied.

## *1. On PDA culture medium*

### Processing steps

- ***Step 1***
  Cut a sample of diseased tissue into several pieces, using sterilized scissors (**Photo VIII–6**).

- ***Step 2***
  Place the sample pieces in a 60-mm petri dish containing a solution of 2.5% sodium hypochlorite. Leave for 3 min to disinfect. Then rinse the disinfected pieces three times with distilled water (**Photo VIII–7**).

- ***Step 3***
  Place the disinfected and washed pieces on sterilized paper towels to dry for 10 min (**Photo VIII–8**).

**Photo VIII–6**

**Photo VIII–7**

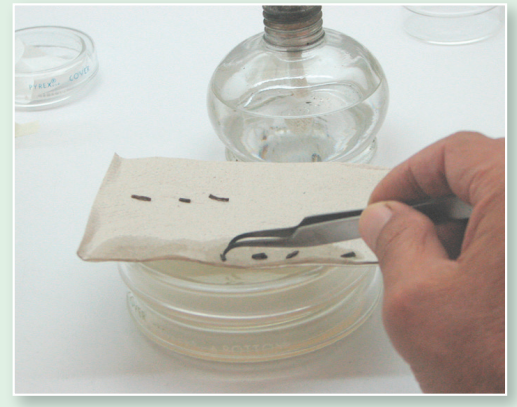

**Photo VIII–8**

Photo VIII–9

Photo VIII–10

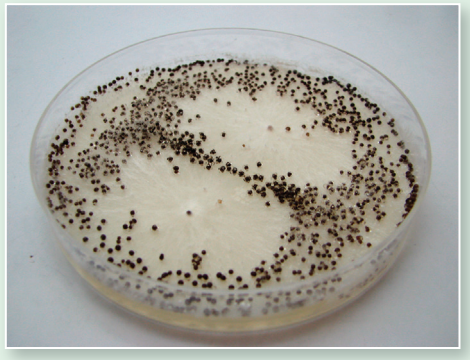

Photo VIII–11

- ***Step 4***
  Once the pieces are dry, use sterilized tweezers to select 3 pieces and culture them in a petri dish containing PDA medium (**Photo VIII–9**).

- ***Step 5***
  Incubate all the samples at 24 °C for 10 days, by this time the fungus will have produced white mycelium (**Photo VIII–10**). This, in turn, will produce sclerotia that, at sight, will appear as black spots (**Photo VIII–11**). Over time, the sclerotia will become brown-colored.

## *2. As a monohyphal isolate*

Because a sample of diseased tissue may carry a mixture of pathogenic strains, the fungus needs to be purified. A genetically pure strain can be obtained through monohyphal isolation, using hyphal tips, as follows:

### Processing steps

- ***Step 1***
  Use a 0.5-cm-diameter punch to cut a disc of fungal growth in PDA culture medium (see *Step 5* above). Transfer the disc to the center of a petri dish containing PDA medium.

- ***Step 2***
  Incubate the dish at 24 °C for 24 to 48 h. Then examine the culture for mycelial growth under the stereoscope in the laminar-flow chamber. Focus on the edges of the colony to identify isolated hyphal tips.

- ***Step 3***
  Once hyphal tips are identified, use a flame-sterilized needle to cut out a piece of agar carrying the tip of a single hypha, as follows:

  – Take care not to touch other hyphae surrounding the selected tip.
  – Transfer the piece of agar with the hyphal tip to a new petri dish containing PDA culture medium. Continue with other dishes, placing only one hyphal tip per dish

- ***Step 4***
  Incubate all the dishes at 24 °C for 10 days. After this time, the fungus should be producing mycelia and sclerotia, which can be observed as small white spots. Over time, they become chestnut- or brown-colored, and will fully mature at 25 days (**Photo VIII–12**). The fungus can then be increased for multiple uses as storage, or inoculation either in the greenhouse or field.

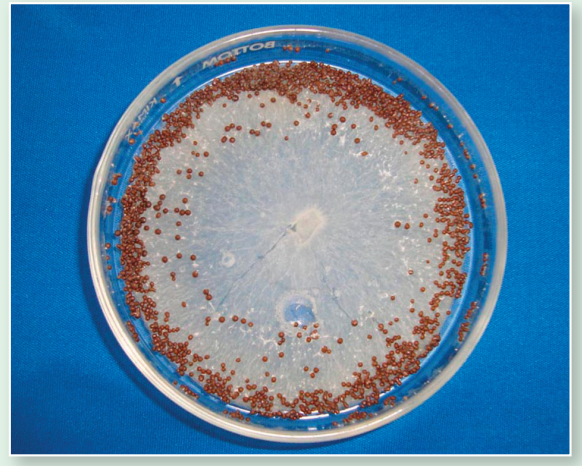

**Photo VIII–12**

## D. Increasing *S. rolfsii*

Sclerotia production from *S. rolfsii* can be improved by supplementing the culture medium with bean flour.

### *1. Preparing agar-bean flour (ABF) medium*

#### Materials

- Bean seeds
- Lidded container
- 15 g granulated agar
- Autoclave
- 1 L distilled water
- Gauze
- Petri dishes
- Rubber bands
- Four 1-L erlenmeyer flasks
- Aluminum foil
- Mill or pestle and mortar

#### *Preparing the bean flour*

- ***Step 1***
  Use a mill or pestle and mortar to mill bean seeds with less than 14% moisture content. Although the product must be powder, the milling does not have to be particularly fine.

- ***Step 2***
  Place the flour in a lidded container and sterilize by autoclaving two times. Once the flour is sterilized, the ABF medium can be prepared.

***Note:*** Whenever bean flour is needed for preparing culture medium, mill and sterilize the flour the day before use.

### *Preparing the medium*

- Mix the agar and bean flour very well.
- Add 1 L of distilled water to this mixture.
- Pour the mixture into 1-L erlenmeyer flasks at 250 mL per flask.
- Place a gauze cover over the mouth of each flask and bind it with a rubber band. Then place aluminum foil over it.
- Sterilize the sealed flasks by autoclaving.
- When the mixture is cool, pour it into petri dishes.

The medium is now ready for culturing the fungus.

## 2. Multiplying monomycelial fungus

- ***Step 1***
  Use a flame-sterilized needle with a piece of agar on its point. Select a stored sclerotium from a glassine envelope (**see *VIII.C.2.*, above**) and pick it up with the agar. Culture the sclerotium into a petri dish containing ABF medium. Repeat this procedure for several petri dishes.

The sclerotium should germinate and the fungus should grow over the entire medium. Six days after culturing, new sclerotia should be forming, appearing first as small white spots that, over time, become either chestnut- or coffee-colored. They reach maturity after 25 days.

- ***Step 2***
  After 25 days, remove the lids from the petri dishes for the sclerotia to dry over 3 days. Once dry, harvest them and store in glassine envelopes or glass tubes at -20 °C.

## E. Inoculating plants in the greenhouse

### *1. Preparing* S. rolfsii *inoculum in a soil-bean flour mixture*

#### Materials

- Soil
- Bean flour
- Distilled water
- Autoclavable trays
- Aluminum foil
- Kraft paper
- *S. rolfsii* sclerotia
- Autoclave
- Balance

## Processing steps

Prepare *S. rolfsii* inoculum on a soil substrate, as follows:

- ***Step 1***
  Prepare the soil substrate as follows:
  - Make, at a 1% rate, a mixture of soil and bean flour, using 10 g of sterilized bean flour to 1 kg of soil.
  - Homogenize the mixture manually by shaking it in a bag.
  - Spread mixture in a tray to form a layer no deeper than 5 cm.
  - Add distilled water at a rate of 50 mL of per kilogram of soil used in the tray.
  - Cover the tray with aluminum foil and then with wrapping paper, and autoclave the whole. After sterilization leave the tray to cool down.
  - Make enough trays for the quantity of inoculum needed.

- ***Step 2***
  Weigh the sclerotia previously harvested and stored (see *VIII.D* above). Spread them uniformly across the soil surface in each tray at 0.2 g (200 mg) of sclerotia to 1 kg of soil.

- ***Step 3***
  Leave the trays out in the laboratory at room temperature. Check them weekly to ensure that the inoculated soil has not been contaminated. After 15 to 17 days, the fungus will have grown sufficiently to colonize the soil with mycelium and produce sclerotia. This soil (**Photo VIII–13**) is used as inoculum in greenhouse tests.

**Photo VIII–13**

Photo VIII–14

Photo VIII–15

Photo VIII–16

## 2. Inoculation

Prepare the planting soil in the greenhouse, as follows:

- Mix soil and sand at a 2:1 ratio.
- Add sterilized bean flour to this soil at a rate of 5 g to 1 kg of soil.
- Add the soil-inoculum mixture (see *Step 3* above) at 3% (**Photo VIII–13**). That is, add 30 g of soil-inoculum mixture to 1 kg of soil.

**Photo VIII–14** shows a greenhouse planting tray with a carrying capacity of 450 kg of soil. This soil received 13.5 kg of soil-inoculum mixture.

Once the inoculated soil is placed on trays, seeds from the bean genotypes to be tested are sown in rows. Depending of the seed availability each assay could be performed using from 6 to 10 seeds per genotype.

Bean germplasm was evaluated on days 7, 14, 21, and 28 after planting (**Photo VIII–15**).

If a trial using different bean genotypes requires large quantities of soil mixture, a portable electric mixer or drum mixer is used (**Photo VIII–16**).

## F. Conserving the fungus for storage

Conserve this fungus as sclerotia, which are obtained during the fungal increase as monohyphal culture (**see *VIII.D.2.* above**). Store sclerotia in glassine envelopes or glass tubes at 20 °C.

## G. Recovering stored fungus

To reactivate growth in *S. rolfsii*, select several sclerotia obtained through monomycelial isolation and stored at -20 °C (**see *VIII.C.2.* above**). Transfer them to ABF culture medium. The sclerotia will germinate and reinitiate fungal growth (**Photo VIII–17**).

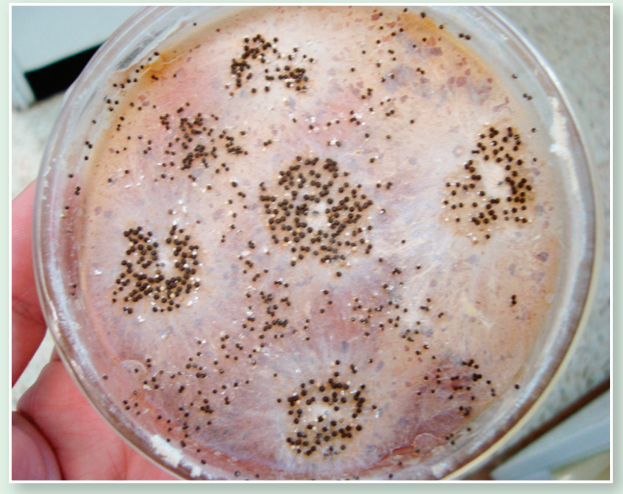

**Photo VIII–17**

# Practical Guide 9

## Fungal mycelium

# Production of fungal mycelium in liquid medium for DNA extraction

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## Contents

# Considerations

Good-quality DNA from phytopathogenic fungi is needed for several procedures such as PCR-Squencing for fungal classification, and another techniques related to genetic characterization. Mycelium is the preferred tissue because it grows more uniformly than other fungal tissues.

Key to extracting good-quality DNA from mycelium is its optimal growth on culture media. This can be achieved by applying best microbiological practices, including:

- Sterilization
- Precise identification of samples
- Harvest the fungus in its exponential growth phase

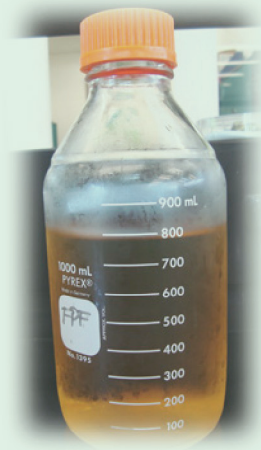
# Procedures

## A. Producing mycelium

### *1. Preparing the liquid medium*

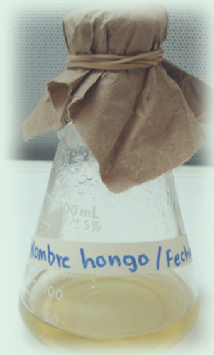
#### Materials

- Fungal isolates for producing mycelium
- Sealing materials for flasks (rubber bands, aluminum foil, kraft paper, cotton wool plugs)
- Twenty 250-mL erlenmeyer flasks
- Shaker
- 20 g dextrose or sucrose
- Autoclave
- 5 g yeast extract
- Laminar-flow chamber
- 5 g peptone
- Sterilized Pasteur pipettes
- 1 L distilled water
- Marker pen


#### Preparation

- To help mixing, heat 1 L of distilled water with the other ingredients until the solution becomes yellow and translucent. This mixture is the liquid medium.
- Pour 50 mL of the liquid medium into each of twenty 250-mL erlenmeyer flasks.




– Seal each flask, using a cotton wool plug, aluminum foil, kraft paper, and a rubber band.
– Sterilize the flasks by autoclaving

The liquid medium can now be used to produce mycelium.

### *Medium inoculation and fungal growth*

Different fungal isolates can be used to produce mycelia, including those that were previously grown in petri dishes containing suitable culture media. Check them for absence of contaminating microorganisms. Conduct the following procedures in a laminar-flow chamber.

## Processing steps

- ***Step 1***
  Write on each of the 20 erlenmeyer flasks, prepared as above, the name of its corresponding isolate to be grown in the liquid medium. Then remove the rubber band, kraft paper, aluminum foil, and cotton wool plug. Because they will be re-used in the next step, place them to one side, ensuring that they will not become contaminated

- ***Step 2***
  Use a Pasteur pipette to draw up 5 mL of liquid medium and deposit them on fungus growing in a petri dish. Scrape the surface of the fungus with the pipette, thus obtaining a suspension of the isolate's mycelium and spores.

- ***Step 3***
  With the same pipette, draw up the suspension and place it in the erlenmeyer flask from which the liquid medium was taken up. Immediately seal the flask, replacing the cotton wool plug, aluminum foil, kraft paper, and rubber band, which had all been kept to one side.

- ***Step 4***
  Place all the flasks on a shaker at 80 rpm at room temperature) up to 10 days. Depending of growth rate of each pathogen, the incubation may vary but visual evaluation can help in deciding when to stop the process. The mycelium should grow sufficiently to enable harvesting.

***Note:*** During shaking, the medium must remain transparent. If it becomes cloudy, it is contaminated. Discard this isolate therefore and repeat the entire procedure.

## B. Harvesting the mycelium

### Materials

- Erlenmeyer flasks containing fungus growing in liquid medium
- Lyophilizer
- Sterilized glass rod
- Screw-cap, glass, test tubes, autoclaved
- Narrow-width masking tape
- Sterilized paper towels
- Marker pen
- Sterilized squares of gauze

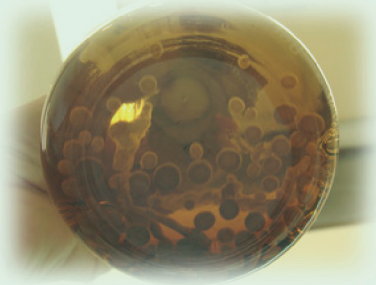


Photo IX–1

Photo IX–2

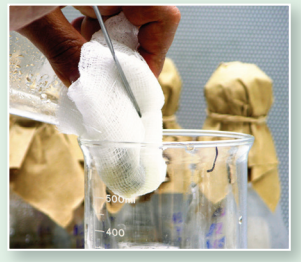


Photo IX–3

Photo IX–4

Photo IX–5

Photo IX–6

– Large glass beaker
– Spatula

## Processing steps

- ***Step 1***
  Remove the rubber band, kraft paper, aluminum foil, and cotton wool plug from each erlenmeyer flask (**Photo IX–1**) carrying a fungal isolate growing in liquid medium.

- ***Step 2***
  Place a 15 x 15-cm square of sterilized gauze or filter paper over the mouth of each flask. Arrange the gauze with the fingers, taking care to handle only the edges. Where necessary, also use a sterilized glass rod. Incline the flask over a large beaker so that the liquid medium passes through the gauze (**Photo IX–2**). The mycelium is captured by the gauze as the medium filters into the beaker (**Photo IX–3**).

- ***Step 3***
  Remove the gauze carrying the mycelium from the flask mouth and place it on two paper towels (**Photo IX–4**). Roll the towels over the mycelium and press with the fingers to extract any remaining liquid medium (**Photo IX–5**).

- ***Step 4***
  Write the isolate's name on a piece of narrow-width masking tape and attach the tape onto a screw-cap test tube in which the corresponding mycelium will be stored (**Photo IX–6**).

- ***Step 5***
  Use a small sterilized spatula to place the dried mycelium in the bottom of the appropriately labeled glass tube (**Photo IX–7**). Screw the cap onto the tube but only half close the tube, that is, do not screw the cap totally down (**Photo IX–8**).

- ***Step 6***
  Take all the loosely closed glass tubes containing mycelium to the lyophilizer (see descriptions of its use in the *Laboratory Practical Guides* I to IV and VI). Dry the mycelium for 24 h (**Photo IX–9**).

- ***Step 7***
  After drying, switch off the lyophilizer, remove the tubes, and tighten their caps. If the mycelium is not to be used immediately for extracting DNA, leave it in the respective tubes and store at -80 °C (**Photo IX–10**).


**Photo IX–7**


**Photo IX–8**

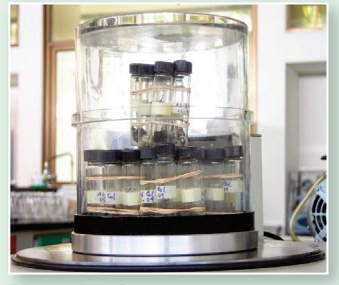


**Photo IX–9**


**Photo IX–10**



# C. Extracting DNA from mycelium

## *1. Solutions for extraction*

The following nine solutions are required for extracting DNA from mycelium. Note, however, that the first three solutions must be made in order to prepare the other six used to extract DNA.

### *Stock solutions for making working solutions*

#### *1M Tris-HCl*

Starting from Trizma® base, molecular weight (MW) = 121.1

**Preparing a 1000-mL solution**

- Dissolve 121.1 g of Trizma® base in 800 mL of distilled water.
- Adjust the pH to the desired value by adding concentrated HCl.
- Complete to 1 L with distilled water and sterilize by autoclaving.
- Store at 4 °C.

#### *5M NaCl*

Molecular weight (MW) = 58.44

**Preparing a 500-mL solution**

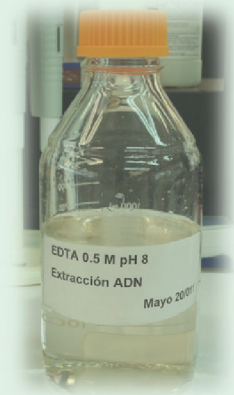
- Dissolve 146.1 g of NaCl in distilled water.
- Sterilize by autoclaving and store at room temperature.

### *0.5 EDTA, pH 8.0*

Molecular weight (MW) = 372.4

**Preparing a 1000-mL solution**

- Dissolve 186.2 g of EDTA in 800 mL of distilled water, using a magnetic shaker.
- Adjust the pH to 8.0 with NaOH (~20 g) and complete the volume to 1000 mL. The EDTA dissolves well when the solution reaches the appropriate pH.
- Sterilize by autoclaving and store at room temperature.




### *Other extraction solutions*

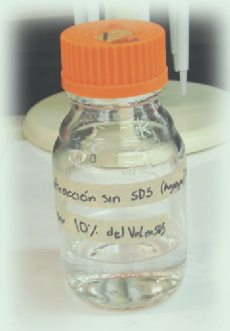
The following six solutions are used directly in extracting DNA from fungal mycelium.

### *7.5 M ammonium acetate*

Molecular weight (MW) = 77.08 ($CH_3$–COO–$NH_4$)

**Preparing a 500-mL solution**

- Dilute 289 g of ammonium acetate in 300 mL of sterilized distilled water. When the acetate is completely dissolved, adjust the volume to 500 mL.
- Sterilize the solution, using 0.22-$\mu$m filters.
- Store at room temperature.




## *Extraction buffer*

Reagents required:

| Reagent | Initial[a] | Volume used (mL) | Final[a] |
|---|---|---|---|
| **Stock solution** | | | |
| NaCl | 5 M | 20 | 0.5 M |
| Tris-HCl, pH 7.5 | 1 M | 40 | 0.2 M |
| EDTA, pH 8.0 | 0.5 M | 4 | 10 mM |
| SDS[b] (2 g) | | | 1% |

a. Initial and final concentrations in 200 mL (see below).
b. SDS = sodium dodecyl sulfate, an anionic, tensioactive compound.

### Preparing 200 mL of buffer

- Dissolve 2 g of SDS in 80 mL of tepid, sterilized, distilled water.
- Add the stock solutions as indicated in the table above, and adjust the volume to 200 mL with sterilized distilled water.
- Keep at room temperature.

## *Proteinase K at 10 mg/mL*

Source: Proteinase K (100 mg). This product can be purchase from several companies, just have in mind the amount that you will use and the expiration date.

### Preparing stocks:

- ***Stock 1 at 20 mg/mL***
  Make a 5 mL solution, using two stock solutions—Tris-HCl at 50 mM and pH 8.0, and $CaCl_2$ at 10 mM—as follows:

- Take 250 $\mu$L of stock solution Tris-HCl at 1 M and pH 7.5
- Add 50 $\mu$L of stock solution $CaCl_2$ at 1 M
- Adjust the volume to 5 mL

- ***Stock 2 at 10 mg/mL***

  - Dilute the 5 mL of Stock 1 with 5 mL of glycerol at 100% to obtain a 50% glycerol solution.
  - Aliquot the solution into 1.5-mL microtubes and store at -20 °C.

The concentration of proteinase K normally used in laboratory work is between 50 and 100 $\mu$g/mL.

### *1X TE, pH 8.0*

Stock solutions used:

| Stock solution | Initial[a] | Volume used (mL) | Final[a] |
|---|---|---|---|
| Tris-HCl, pH 8.0 | 1 M | 5 | 10 mM |
| EDTA, pH 8.0 | 0.5 M | 1 | 1 mM |

a. Initial and final concentrations in 500 mL (see below).

**Preparing a 500-mL solution**

- Adjust the volume of the two stock solutions (6 mL) to 500 mL with sterilized distilled water.
- Sterilize the solution by pouring through 0.22-$\mu$m filters.
- Aliquot into 1.5-mL microtubes and store at 4 °C.



### *RNase A at 1 mg/mL*

Source: RNase A can be purchase from several companies, just have in mind the amount that you will use and the expiration date.

**Preparing stocks:**

- ***Stock 1 at 10 mg/mL***
  - Resuspend all the enzyme contents in 10 mL of the following solution: 10 mM Tris-HCl, pH 7.5 (100 $\mu$L of 1 M Tris-HCl) + 15 mM NaCl (37.5 $\mu$L of 1 M NaCl).
  - Heat this suspension at 100 °C for 15 min.
  - Leave to cool to room temperature and take aliquots.

- ***Stock 2 at 1 mg/mL***
  - Dilute 100 $\mu$L of Stock 1 with 900 $\mu$L of a solution of 10 mM Tris-HCl, pH 7.5 + 15 mM NaCl (37.5 $\mu$L of 1 M NaCl).
  - Make aliquots and store them at -20 °C.

### *Sodium acetate at 3 M, pH 5.2*

Molecular weight (MW) = 82.03 (anhydrous) ($CH_3-COO-Na$)

**Preparing a 100-mL solution**

- Dissolve 24.61 g of sodium acetate in 80 mL of distilled water.
- Adjust the pH to 5.2 with glacial acetic acid and complete the volume to 100 mL.
- Sterilize by autoclaving and keep at room temperature.

## 2. DNA extraction

Take the following steps to extract DNA from mycelia of the pathogenic fungi described in the *Laboratory Practical Guides* I and III to VIII:

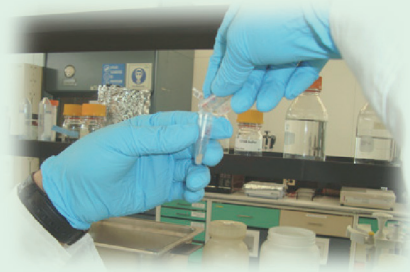
### Processing steps

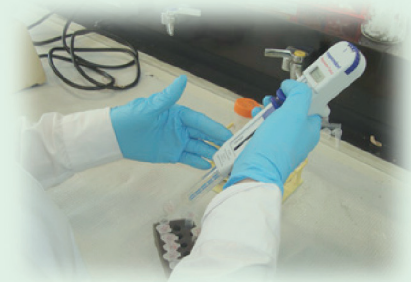
- ***Step 1***
  Use a water bath to pre-heat the extraction buffer to 65 °C.

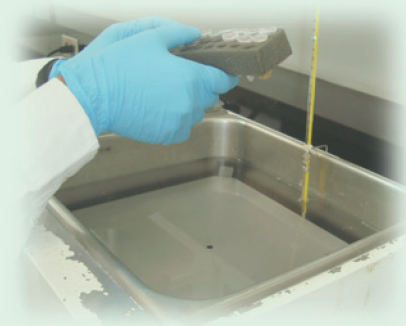
- ***Step 2***
  Add proteinase K at 10 mg/mL to the buffer until a final concentration of 30 μg/mL is obtained. That is, add 3 μL of proteinase K for each milliliter of buffer. This is equivalent to using 2.1 μL of proteinase K for every 700 μL of buffer.

- ***Step 3***
  Macerate either fresh or stored mycelium, using liquid nitrogen. Transfer the macerated mycelium to 1.5-mL eppendorf tubes, filling them to the 100- or 150-μL mark in the tube.

- ***Step 4***
  To the mycelium in each 1.5-mL tube, add 700 μL of extraction buffer with proteinase K, as prepared in *Steps 1* and *2* above. Homogenize the mixture by using either a vortex or tube mixer, and incubate in a bain-marie at 65 °C for a minimum of 1 h.







- ***Step 5***
  Mix by inverting manually every 20 or 30 min.

- ***Step 6***
  This step has two options:

  a. After incubation (*Step 4* above), add 0.5 volumes of previously prepared ammonium acetate at 7.5 M and mix by inverting the tube. Incubate at room temperature for 10 min.
  b. After incubation (*Step 4* above), mix again and add an equal volume (700 $\mu$L) of a solution of phenol, chloroform, and isoamyl alcohol at 25:24:1. Mix with a vortex.

***Note:*** The phenolic solution must be handled in an extractor chamber to prevent inhalation of vapors from the process.

- ***Step 7***
  Centrifuge at 12,000 rpm for 10 min at room temperature.

- ***Step 8***
  Retrieve the supernatant, taking care not to draw up the interface. Transfer it to a fresh 1.5-mL eppendorf tube.

- ***Step 9***
  Add an equal volume of a solution of chloroform and isoamyl alcohol at 24:1. Centrifuge at 12,000 rpm for 10 min at room temperature.

***Note:*** The solution must be at room temperature at the moment of use and must be handled in an extractor chamber to prevent inhalation of vapors from the process.

- ***Step 10***
  Recover the supernatant and transfer it to a fresh 1.5-mL eppendorf tube.

- ***Step 11***
  *Optional step*: Add 3 M sodium acetate (pH 5.2) at a ratio of 1:10 of supernatant volume. Homogenize manually by inverting.

- ***Step 12***
  Add cold isopropanol at a ratio of 1:1. Homogenize by gently inverting the tube manually.

- ***Step 13***
  Incubate overnight at -20 °C.

- ***Step 14***
  Centrifuge at 14,000 rpm for 10 min at room temperature. Discard the supernatant by inverting the tube, taking care not to lose the pellet of DNA.

- ***Step 15***
  Wash the pellet with 800 $\mu$L of 70% ethanol, leave to cool, and centrifuge at 14,000 rpm for 5 min.

- ***Step 16***
  Discard the supernatant and dry the pellet by inverting the tube at room temperature.

- ***Step 17***
  Dilute the pellet in about 100 $\mu$L of previously prepared 1X TE buffer. Add 2 $\mu$L of RNase A at 1 mg/mL. Incubate the whole at 37 °C for a minimum of 1 h.

  ***Recommendation:*** Resuspend the pellet overnight at 4 °C with 1X TE buffer. The next day, add RNase A and then incubate at 37 °C.

- ***Step 18***
  Store the DNA at -20 °C.

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**Bean Plant Pathology**

***and***

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