

Inoculum inoculation and media preparation of anthracnose, caused by *Colletotrichum lindemuthuanum*

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Depending on the race of anthracnose you are working with different races require different media. For example: race 7 sporulates very well on Potato Dextrose Agar (PDA), while race 73 sporulates on Mathur Agar. When using a race for first time, plate the race on both media to determine which medium is better for fungal sporulation.

Media Preparation

Potato Dextrose Agar

To prepare Potato Dextrose Agar (PDA) media:

- 1) Weigh 39g of PDA
- 2) Add 1000mL distilled water.
- 3) Stir using magnetic plate.
- 4) Autoclave for 25 minutes.
- 5) Under sterilized laminar flow hood, let the media cool down then pour into sterilized Petri dishes. Let the plates solidify.

Note: to avoid contamination, add ampicillin 50µg/ml (it is option) to the media before pouring into Petri dishes. This helps to avoid bacterial contamination.

- 6) Store plates in refrigerator.

Mathur Agar

This medium is made up of the following reagents: dextrose, magnesium sulfate, potassium phosphate, neopeptone, yeast extract, and agar.

How to prepare 500ml of Marthur media: weigh the following ingredients:

- Dextrose 4.0g
- Magnesium Sulfate 1.25g
- Potassium Phosphate 1.35g
- Neopeptone 1.2g
- Yeast Extract 1.0g
- Agar 8.0g

- 1) Measure 250ml of distilled water and pour in beaker with a stir bar

- 2) Add all reagents (dextrose, magnesium sulfate, potassium phosphate, neopeptone and yeast extract) except the agar and stir using a magnetic stir plate until all the reagents are dissolved.
- 3) Take another beaker, pour 200ml of distilled water and add the agar.
- 4) Heat the agar and distilled water in the microwave until boiling.
- 5) Add the solution from the step 2 to the pre-boiled agar and stir them all together using magnetic plate until the agar dissolves.
- 6) Autoclave for 25 minutes. See the above for instruction for pouring the media.
- 7) Store the plates in refrigerator.

Plating the fungus

Using laminar flow hood, place the fungus on medium and place the plates in incubator (22° -25°C) upside down to grow. This is referred to as the base plate. After five days, the fungus is ready to replate from the base plate to new plates either by: 1) cutting small pieces of agar with the fungus and placing them onto a new plate or 2) streaking the spores onto new plates if the fungus is sporulating well. In ten days the fungus will be ready to inoculate the plants. When the fungus begins sporulating, you can plant your seeds and in meantime make more plates of the fungus. Be sure that your fungal plates to be less than one month old from the date you plate when you want to inoculate the plants.

Inoculum and inoculation preparation:

To make 100ml spore suspension

- 1) Take a beaker, add 100 ml of distilled water and 10 drops of Tween20 using Pasteur pipet stir using magnetic stir plate
- 2) Put 10 ml of water solution on fungal plate and scrape the spores using spatula.
- 3) Place the spore mixture in a small beaker put stir bar and stir for 10 minutes.
- 4) Filter the mixture using cheese cloth and funnel and quantify the spores using Hemacytometer and light microscope.
- 5) Adjust the spore suspension to ideal concentration of 1.2×10^6 spores/ml
- 6) Inoculate the bean plants by spraying the spore suspension starting from the abaxial to the adaxial of the leaves
- 7) Place the plants in the mist chamber for 48 hours and 100% Relative Humidity.
- 8) Take the plants out from mist chamber.
- 9) Water the plants once a day while you are waiting the symptoms to show.

10) Evaluate after 7 days from the inoculation..

If the fungus does grow on either Potato dextrose agar or Mathur agar, then another alternative is **bean pod agar media**.

How to prepare bean pod agar media

Bean Pod Agar Media

Items needed to make this media:

- 500g of green bean pods
- 16g of Agar
- 1L distilled water

Pod and media preparation

- 1) Cut off the two ends of the bean pod
- 2) Wash the pods and autoclave them 15-20min.
- 3) Prepare the water agar(16g of agar in 1L of distilled water) and autoclave for 20min.
- 4) Pour 3mL of water Agar in each tube (clean tubes)
- 5) Place the bean pod upright in each tube
- 6) Place cotton ball and cap
- 7) Autoclave them for 20 min.
- 8) Wait until media solidifies (30 min)

Inoculum preparation

Since the fungus is already grown in Petri dish, but not sporulating on artificial media bean pod agar may provide nutrients needed for sporulation.

- 1) Using a sterile needle cut small pieces of agar with the fungus from the corner of the plates and place agar plugs in the tubes with sterile bean pods
- 2) Place the tubes in the incubator 25°C
- 3) Monitor after 3-4 days or wait until the fungus fully grows
- 4) Replate the fungus into new tubes containing media with pod
- 5) The fungus will be ready to inoculate in two weeks

Inoculation procedure

- 1) Add 100 drop of Tween20 in 1L of distilled water
- 2) Take spatula and remove the pods from the tubes

- 3) Place them in a beaker and add 200ml of distilled water
- 4) Stir using magnetic stir plate until spore suspension is formed
- 5) Place cheesecloth into the top of a funnel
- 6) Place the funnel with cheesecloth into the flask and pour the spore suspension.
- 7) Count the spore concentration using Hemacytometer and adjust the concentration of spores to 1.2×10^6 spores/ml
- 8) Inoculate your plants as previously described. Re-isolate the fungus from inoculated plants.

Fungal re-isolation

Item needed for this procedure:

- 1% bleach (chlorox)
- 75% ethanol
- sterilized filter and paper towels
- sterilized distilled water
- clean beaker

Wipe the bench with ethanol before use. Using laminar flow hood, flame the knife and forceps many times to be sure that they are sterile. Cool them before using them. Spread paper towel and put the infected tissues on the paper. Cut the tissues into small pieces of .5 cm long. Place the tissues into small beaker add 10ml bleach stir for 2 minutes. Throw away the bleach and then add 10ml of ethanol and stir for 2 minutes. Throw away the ethanol and rinse the tissues with sterilized distilled water just once. Flame the forceps again. Put the tissues on the filter paper to take excess water and dry them. Place the tissues on the media. Place the plates in the incubator upside down to grow.

After four days the fungus should show some growth. Re-plate the fungus into a new plates.

Long-term storage

For future use, isolates can be stored at -20°C . To do so you must have the following items ready:

- 1) Not contaminated and sporulating fungal plate
- 2) Sterilized filter papers(cut the filter paper 2cm long)
- 3) One or two plates of media depending the isolate(PDA for 7 and

MA for 73)

4) Needle and forcep

Using laminar hood flow, sterilize the forcep several times by dipping in ethanol and flaming. Let it cool. Place pieces of sterilized filter papers on the media (15 pieces). Take sterilized needle and cut small piece of agar with fungus. Place the agar on the filter paper, then using the needle divide this piece into smaller pieces and place a tiny piece on each filter paper. Cover the plate, write the name of isolate (fungus) and the date.

Place the plate upside down in the incubator. After five days to one week the fungus will cover the filter papers. Using laminar hood flow, sterilize forcep and let it cool down. Peel gently the filter papers and place them in disposable sterilized Petri dish (no media). Place the Petri dish plate in the incubator to dry the filter papers. After 4-5 days the filter papers are dry. In order to place the isolate (fungus) in the freezer, place the fungus in sterilized aluminum foil using sterilized forcep then fold foil, then place the aluminum in shoot bag. Write the name of fungus or isolate number like *CL.1.0* and the date prepared. It is also nice if you could arrange your collection based on the country from which the isolate was collected like (US1.0). Place the fungus in the freezer (-20°C)