Canadian Collection of

ARBUSCULAR MYCORRHIZAL FUNGI - CCAMF

Protocols for in vivo and in vitro cultures

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada
Canada

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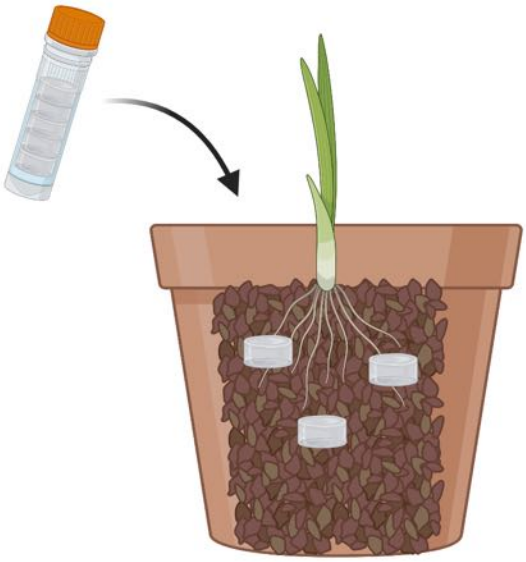
Protocol 1: Start an in vivo culture with spores from an in vitro culture

Step 1. Pot preparation

Soil is humidified and autoclaved twice for one hour.
Clean pot in soap and then in bleach solution for an hour, rinsed thoroughly.

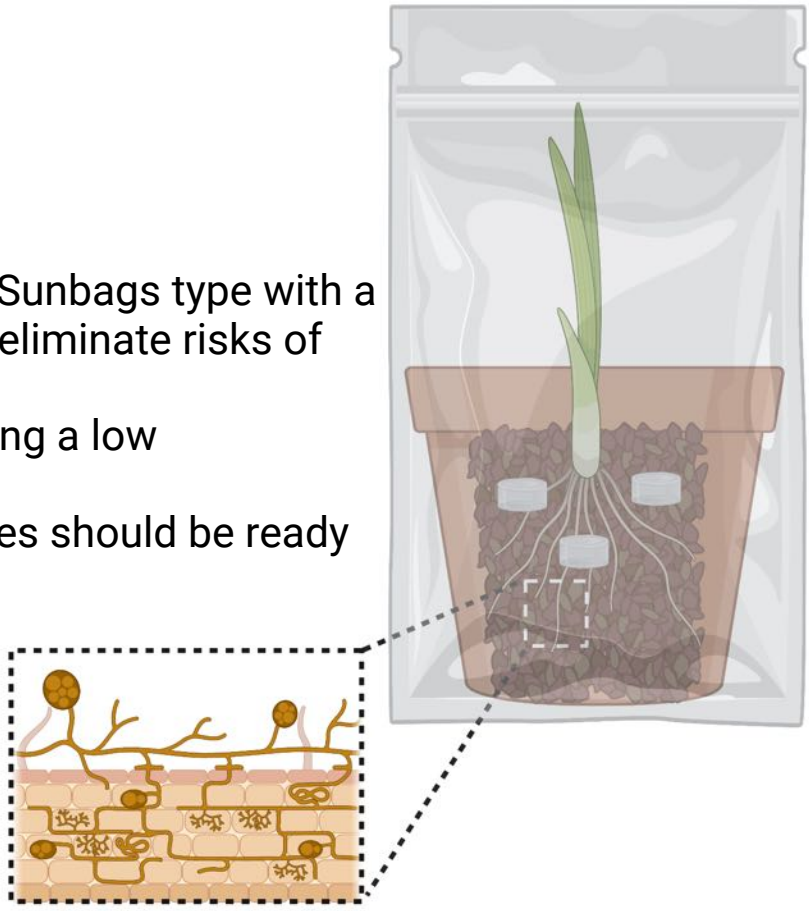


Step 2. Inoculation

Water the soil. Put the gel plugs from the vial in the soil mixture, 2-3 cm below the surface so they don't dry out.
Plant the host. Use a plantlet that was grown in sterilized soil.

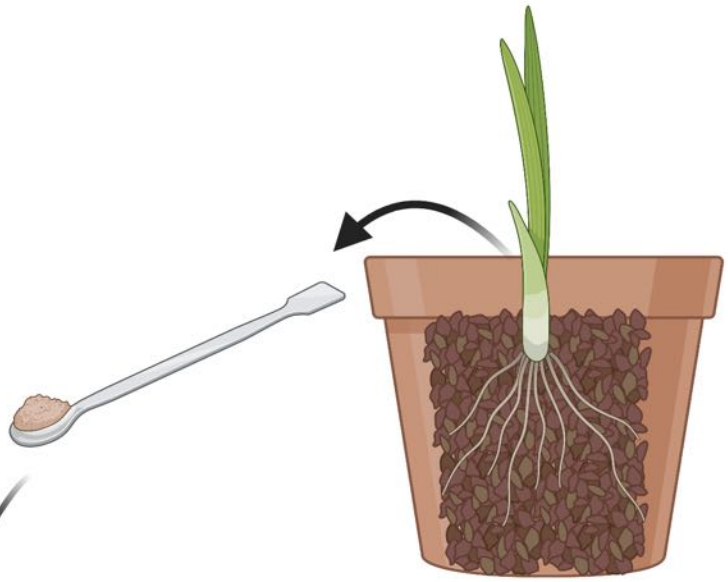
Step 3. Cultivation

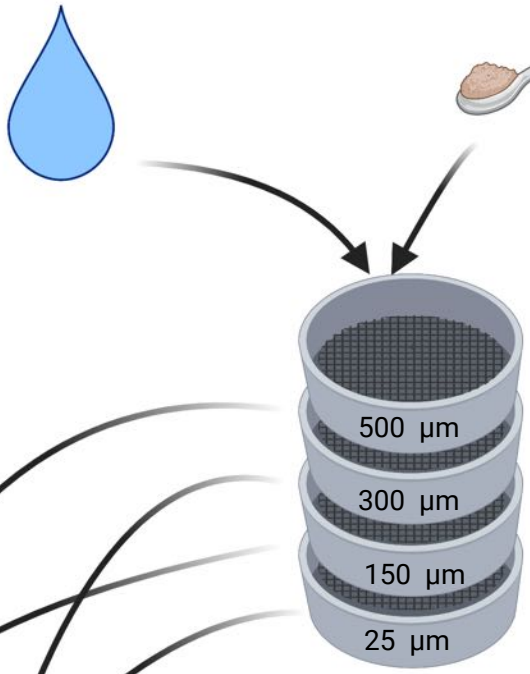
Enclose the pot in a sealed bag (Sunbags type with a membrane for gas exchange) to eliminate risks of aerial contamination.
Water and fertilize as needed using a low phosphorous fertilizer.
Depending on plant growth, spores should be ready to harvest in 6-8 months.



Protocol 2: Extract spores from an in vivo culture

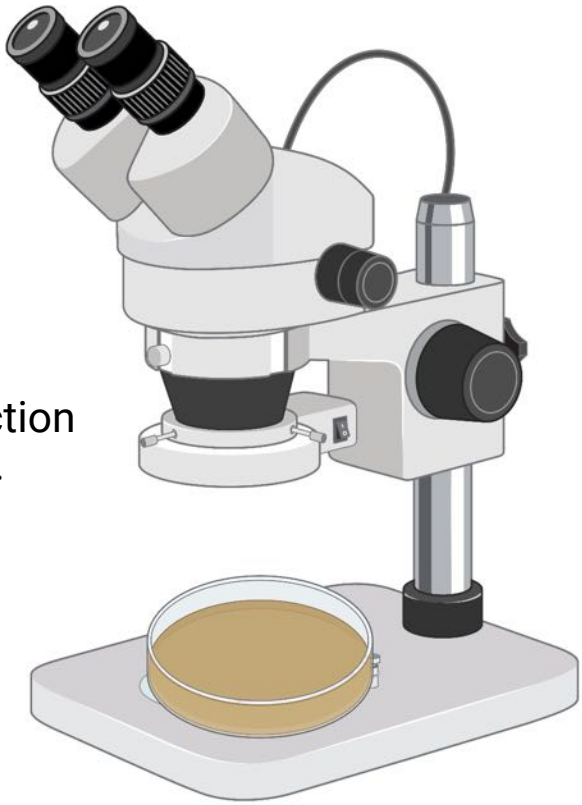
Step 1. Soil sampling
Take a sample of soil from a culture established for at least 6-8 months.





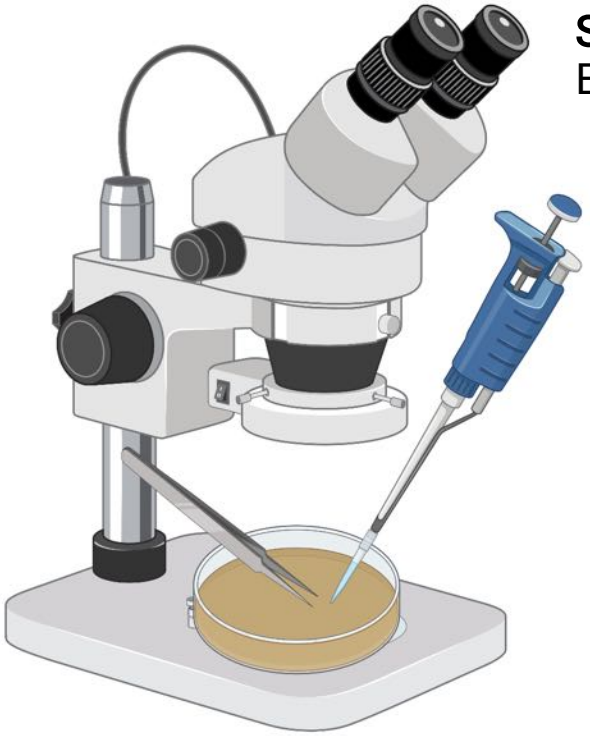
Step 2. Wet sieving
Under running water, strain the soil through four stacked sieves.

Step 3. Transfer
Using water, transfer each fraction of soil into a transparent plate.

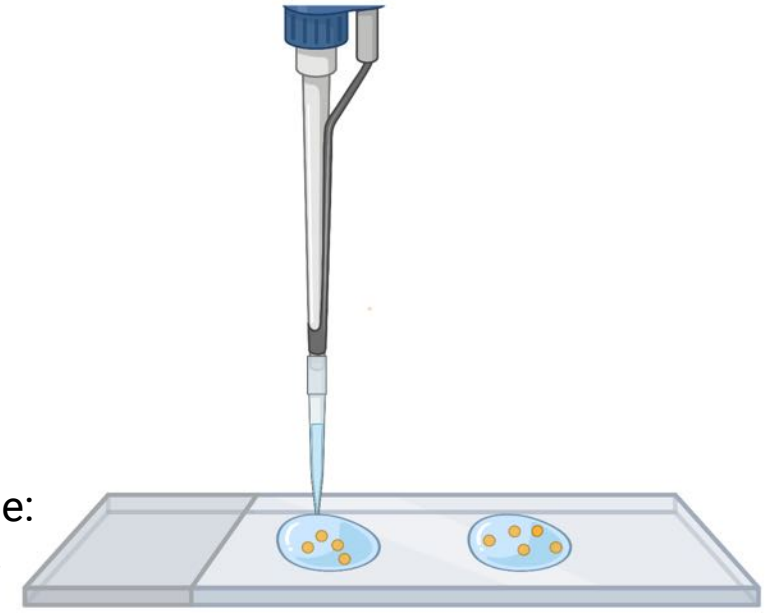


Step 4. Observation
Look at each plate under a stereoscope.
Use tweezers and a pipet to extract spores.

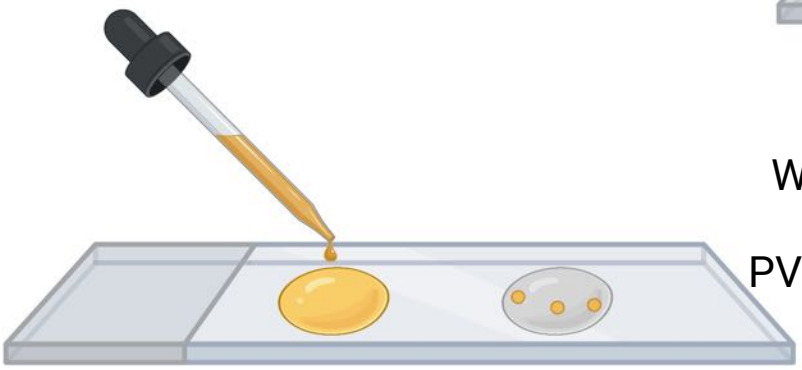
Protocol 3: Mounting a spore slide for microscope analysis



Step 1. Sample spores
Extract the spores from a soil fraction.



Step 2. Transfer spores
Transfer the spores to a microscope slide: about two droplets of water with spores.



PVLG+Melzer PVLG

Step 3. Add mounting buffer
When the water has evaporated, put one drop of reagent on top of the spores. PVLG* on one side and PVLG + Melzer on the other side.

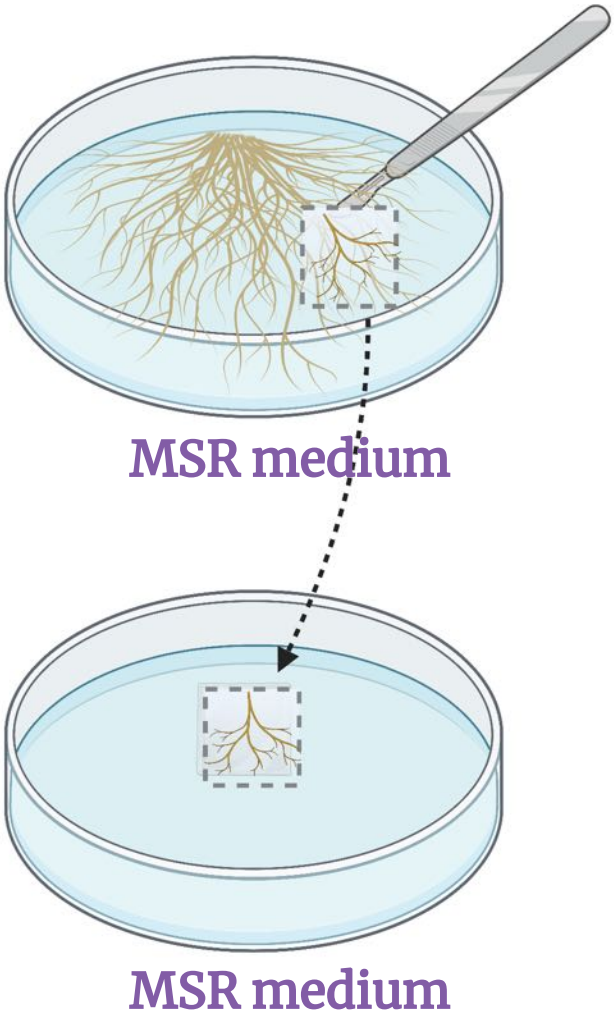
Step 4. Cover slips
Add the cover slips, and tap their surface to squash the spores.
Let the slide dry for 24 hours before observation.

**polyvinylalchohollactic acid-glycerol*

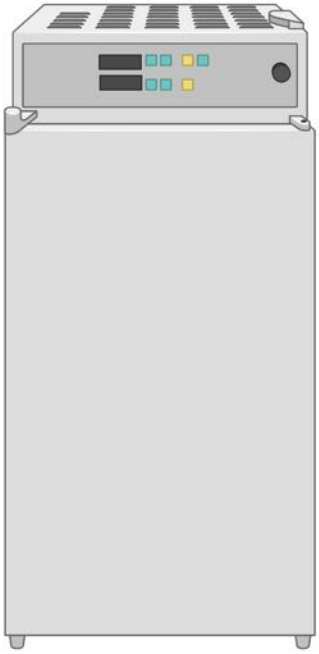
Protocol 4: Maintain monoxenic cultures of Ri T-DNA* transformed roots

All manipulations are done with sterilized tools in a laminar flow hood. Roots should be subcultured once a month.

Step 1. Sample roots
Cut a piece of Modified Strullu and Romand (MSR) medium from a fresh culture (approximately one month old). Choose roots that are light coloured and branched.



Step 2. Transfer
Transfer the piece of medium containing the root sample on fresh MSR medium.



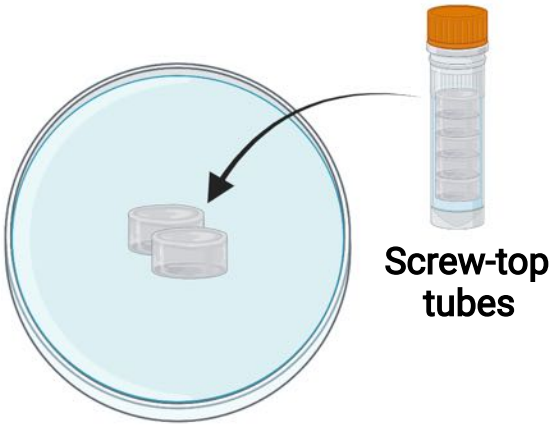
Step 3. Incubation
Incubate the Petri dishes in the dark at 26°C. Depending of the root geotropism, the Petri dishes might be incubated upside down.

**Rhizogene induced transfer DNA*

Protocol 5: Start an in vitro culture from sterile spores

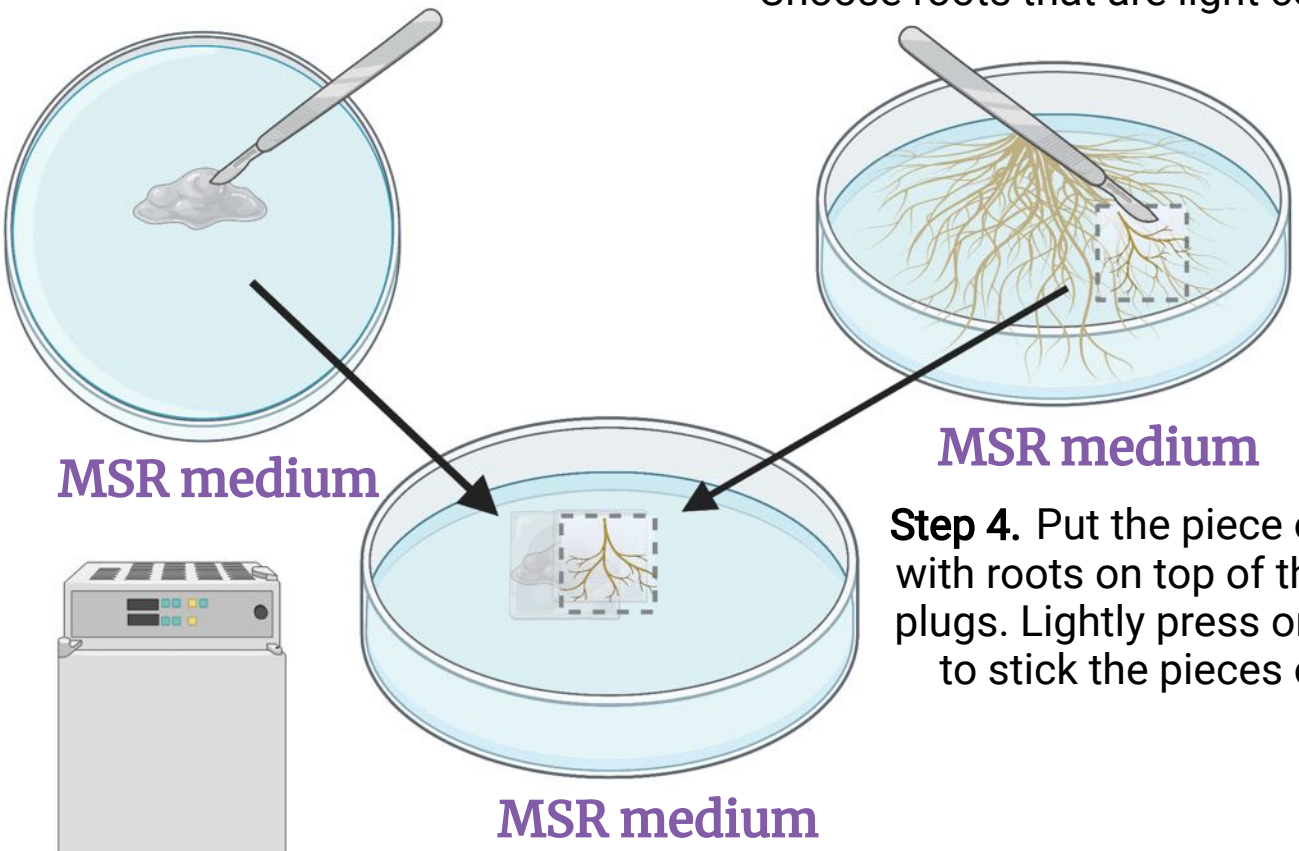
All manipulations are done with sterilized tools in a laminar flow hood

Step 1. Put the gel plugs on the Modified Strullu and Romand (MSR) medium. Up to four cultures can be prepared with the gel plugs contained in a tube.



Step 2. Cut the gel plugs in smaller pieces.

Step 3. Cut a piece of medium from a monoxenic root culture (one month old). Choose roots that are light colored and branched.



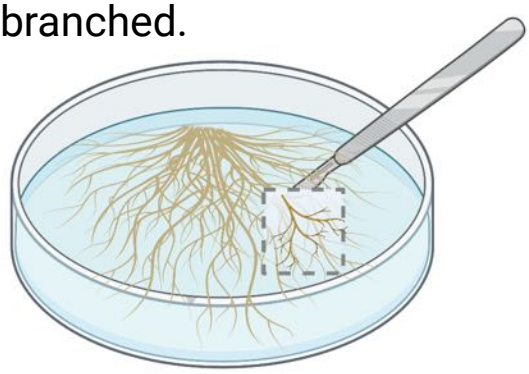
Step 4. Put the piece of medium with roots on top of the crushed plugs. Lightly press on the roots to stick the pieces of medium together.

Step 5. Incubate the Petri dishes in the dark at 26°C.

Protocol 6: Start an in vitro culture from another culture

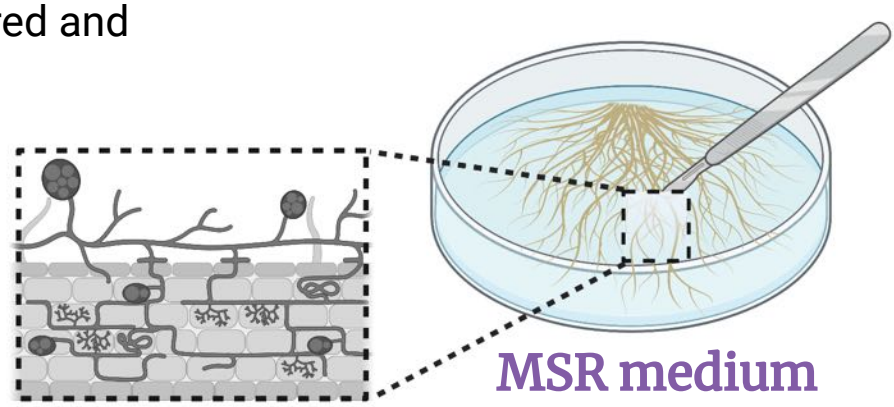
All manipulations are done with sterilized tools in a laminar flow hood

Step 1. Cut a piece of Modified Strullu and Romand (MSR) medium from a **Ri T-DNA* root culture** (one month old). Choose roots that are light coloured and branched.



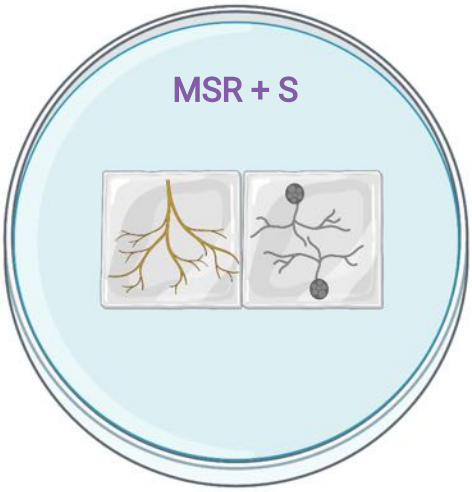
MSR medium

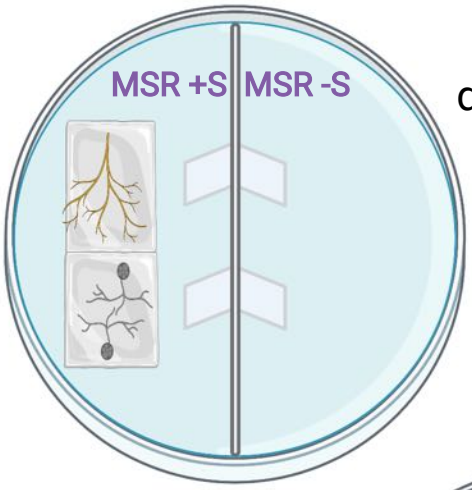
Step 2. From an inoculated culture, cut a piece of medium containing many healthy spores (with lipids).



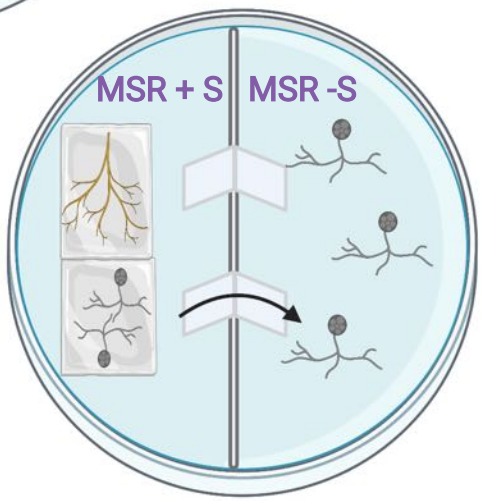
MSR medium

Step 3. Transfer the roots and spores in a mono- or bi-compartmented Petri dish.





In bi-compartmented Petri dishes, put the spores and the roots on the MSR + sucrose (S) side. The hyphae and spores will cross the paper bridges and sporulation will occur in the compartment without sucrose.



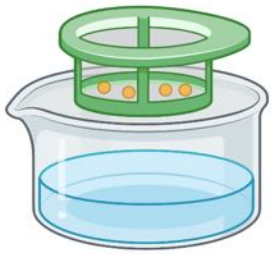
Step 4. Incubate the Petri dishes at 26°C in the dark.

**Rhizogene induced transfer DNA*

Protocol 7: Spore sterilization (part 1)

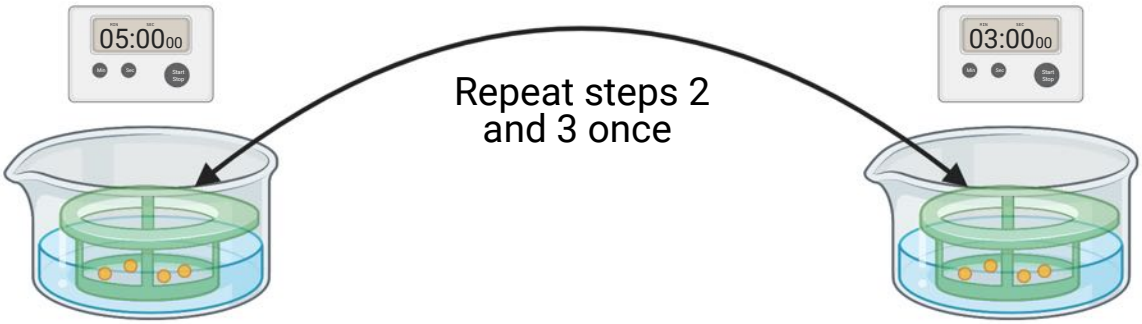
All manipulations are done with sterilized tools in a laminar flow hood

Step 1. In a laminar flow hood, pipet the spores in a 40 µm strainer on top of a glass plate.



Step 2. Immerse the strainer in sterile distilled water. Gently shake for 5 minutes changing the water twice. Discard the liquid.

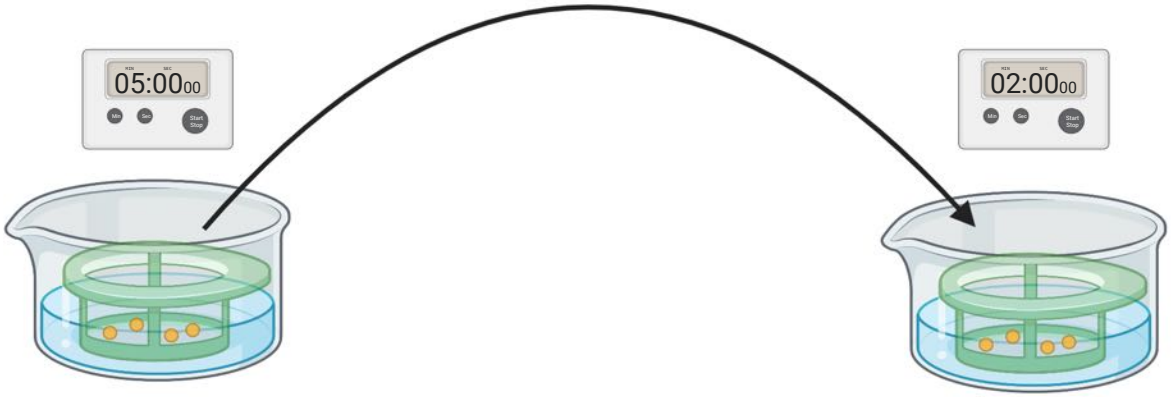
Step 3. Immerse in 2% Chloramine T solution with 1-2 drops of Tween Gently shake for 3 minutes. Discard the liquid.



H_2O $C_7H_7ClNO_2SNa$

Step 4. Immerse the strainer in sterile distilled water.
Gently shake for 5 minutes changing the water twice. Discard the liquid.

Step 5. Immerse in the streptomycin and gentamicin antibiotics solution (0.2 ml / 10 ml of stock solution*)
Gently shake for 2 minutes.



H_2O $C_{21}H_{39}N_7O_{12}$ $C_{21}H_{43}N_5O_7$

*0.2 g/ 20 ml streptomycin
0.1 g / 20 ml gentamicin

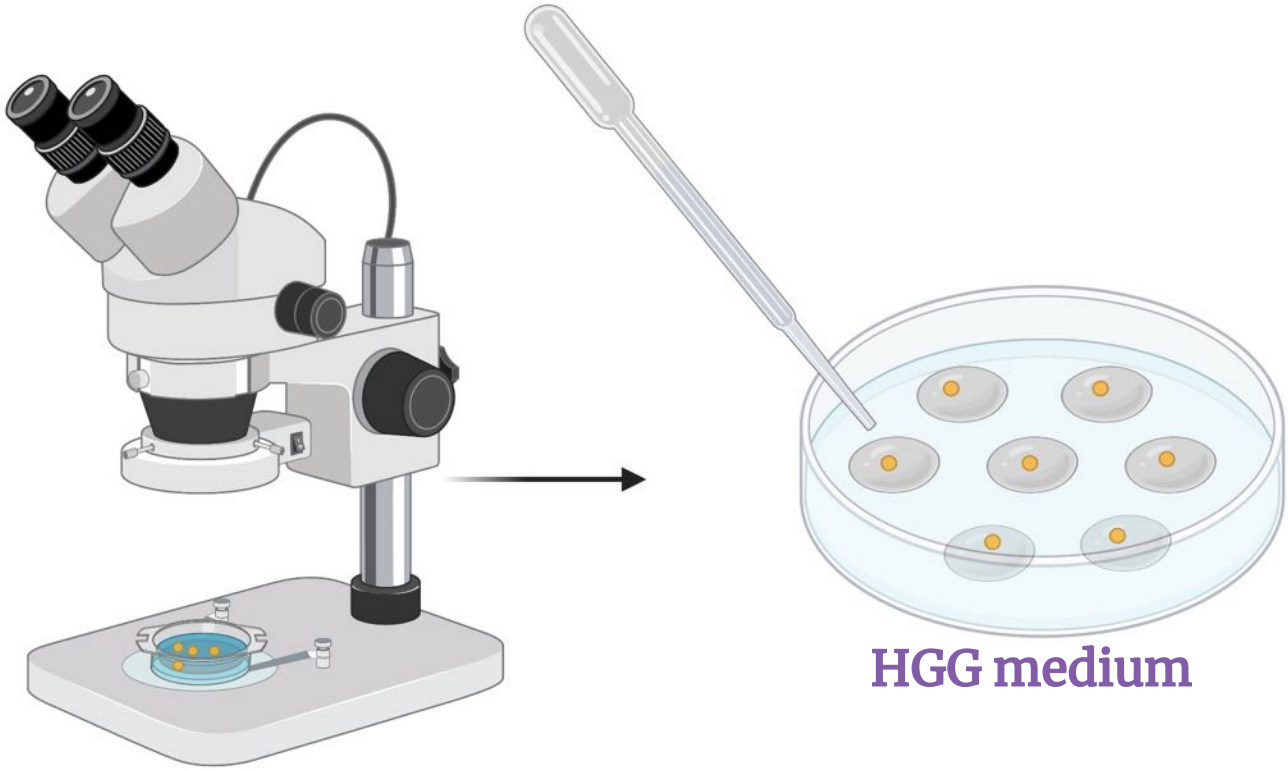
References :

Bécard G and Fortin JA (1988). Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytologist 108, 211–218.

Declerck S, Strullu D-G and Fortin JA. (2005). In Vitro Culture of Mycorrhizas. Springer, Berlin Heidelberg New York, 388 p.

Protocol 7: Spore sterilization (part 2)

Step 6. Transfer spores one by one with a droplet of antibiotics in Petri dish with Hydrated Gellan Gum (HGG) medium, pH 7. Once the droplets have evaporated, close the Petri dish and incubate in the dark at 26°C with 2.5% CO_2. **Check daily for contaminants and remove them quickly.**

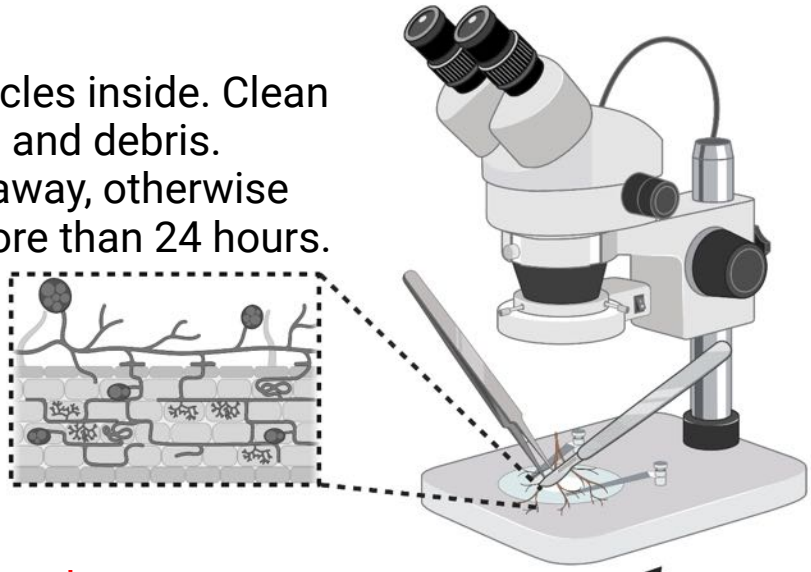


Step 7. As soon as a spore germinates, transfer it on Modified Strullu and Romand (MSR) medium and put pieces of roots around the spore. Incubate the Petri dish in the dark at 26 °C.

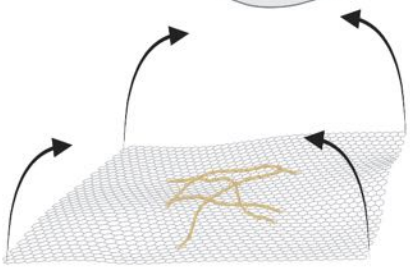
The piece of HGG medium with the spore should be placed upside down on the Petri dish without disturbing the spore or breaking the germination hyphae.

Protocol 8: Sterilize roots with vesicles (part 1)

Step 1. Choose healthy roots with vesicles inside. Clean the roots and remove damaged pieces and debris. Ideally you should sterilize them right away, otherwise you can refrigerate the roots but no more than 24 hours.



Step 2. From there, all manipulations are done with sterilized tools in a laminar flow hood. Place the roots in a nylon mesh (100 µm). Fold the corners of the mesh to create an envelope, with the roots sealed inside.



Step 3. Put the folded mesh in an histology cassette.

Step 4. Immerse the cassette in sterile distilled water. Gently shake for 5 minutes. Stop and change the water twice. Discard the liquid.

H_2O

Step 5. Immerse the strainer in 95% Ethanol for 10 seconds. Discard the liquid.

C_2H_5OH

Protocol 8: Sterilize roots with vesicules (part 2)

All manipulations are done with sterilized tools in a laminar flow hood

Step 6. Repeat step 4 (distilled water rinse).

Step 7. Immerse in a freshly made and filtered 6% calcium hypochlorite solution. Gently shake for 1 minute. Discard the liquid.

$Ca(ClO)_2$

Step 8. Repeat step 4 (distilled water rinse).

Step 9. Immerse in a 2% Chloramine T solution with 1-2 drops of Tween. Gently shake for 5 minutes. Discard the liquid.

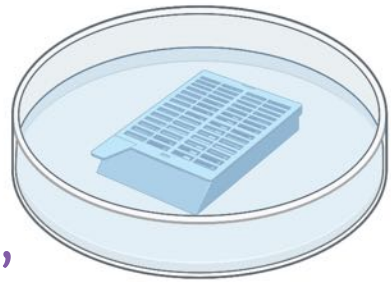
$C_7H_7ClNO_2SNa$

Step 10. Repeat step 4 (distilled water rinse).

Step 11. Repeat steps 9 & 10 (Chloramine T + distilled water).

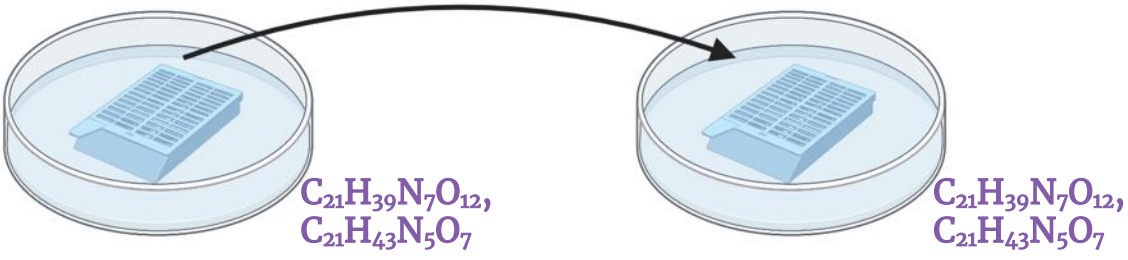
Step 12. Immerse in the streptomycin and gentamicin antibiotic solutions (0.2 ml / 10 ml of stock solution*). Gently shake for 6 minutes.

*0.2 g/ 20 ml streptomycin
0.1 g / 20 ml gentamicin



$C_{21}H_{39}N_7O_{12}$, $C_{21}H_{43}N_5O_7$

Step 13. Transfer the cassette to a Petri dish with fresh antibiotics solution.



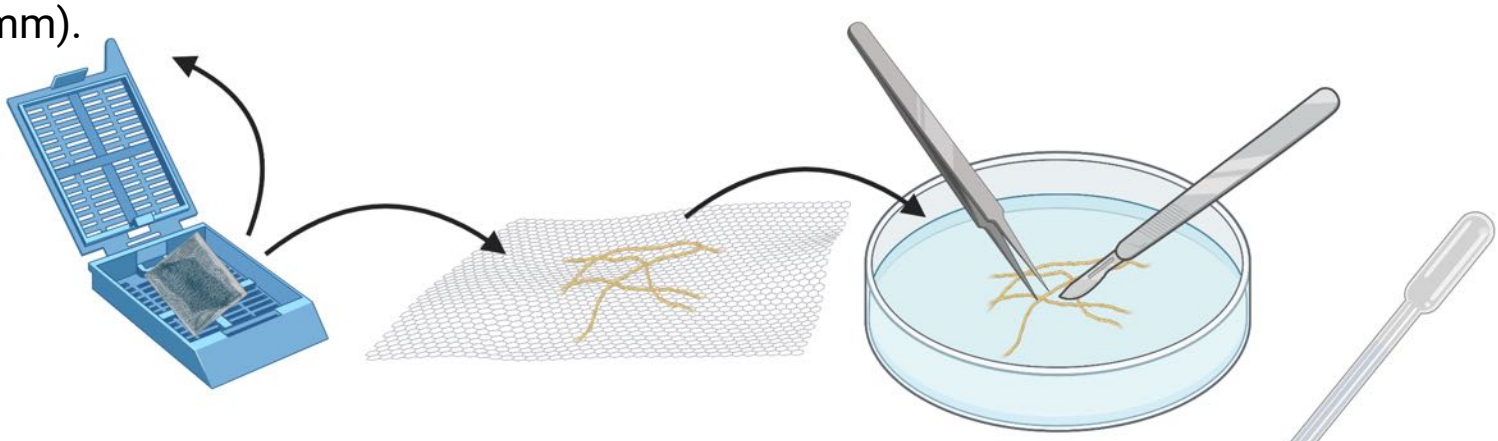
$C_{21}H_{39}N_7O_{12}$, $C_{21}H_{43}N_5O_7$

$C_{21}H_{39}N_7O_{12}$, $C_{21}H_{43}N_5O_7$

Protocol 8: Sterilize roots with vesicules (part 3)

All manipulations are done with sterilized tools in a laminar flow hood

Step 14. Open the cassette, transfer the roots out. Cut the roots in the smallest pieces possible with a scalpel and tweezers (approximately 1-2 mm).



Step 15. Transfer the pieces of roots one by one with a droplet of antibiotics in Petri dish filled with Hydrated Gellan Gum (HGG) medium, pH 7. Once the droplets have dried, close the Petri dish and incubate in the dark at 26°C with 2.5% CO_2. **Check the Petri dishes daily for contaminants and remove them quickly.**

Step 16. As soon as a vesicule germinates, transfer the piece on Modified Strullu and Romand (MSR) medium and put pieces of monoxenic root cultures around the sterilized root. Incubate the Petri dish in the dark at 26°C.

The piece of medium with the germinating root should be placed upside down on the Petri dish without disturbing the vesicules or breaking the germination hyphae.