Inoculation and Growth with Mycorrhizal Fungi

Mireille Chabaud[1], Maria Harrison[2], Fernanda de Carvalho-Niebel[1], Guillaume Bécard[3] and David G. Barker[1].

[1]Laboratoire des Interactions Plantes-Microorganismes, INRA-CNRS, BP 52627, 31326 Castanet Tolosan Cedex, France.

[2]Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY 14853-1801, U.S.A.

[3]Signaux et Messages Cellulaires chez les Végétaux, UMR 5546, CNRS-Université Paul Sabatier, BP 41617, 31326 Castanet Tolosan Cedex, France.

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Introduction

The development of *M. truncatula* as a model plant for studying root symbioses has led to the establishment of various inoculation protocols for arbuscular mycorrhizal (AM) fungi which will be presented in this chapter (see also chapter "Symbiotic interactions – Arbuscular mycorrhiza"). Following spore germination in the soil, AM hyphal branching (a prerequisite for successful root infection) is stimulated by strigolactone exudates generated by the host root (Akiyama *et al.* 2005, Besserer *et al.* 2006). Infectious hyphae which penetrate the root

are initiated from appressorial structures which differentiate on the epidermal surface. After traversing the outer layers of the cortex these infection hyphae penetrate inner cortical cells and differentiate into arbuscules, which are the preferential sites for metabolic exchange between the two partners. In addition to initial (apoplastic) infection through the root epidermis, subsequent intercellular colonisation along and within the inner root cortex leads to the propagation of fungal infection in the root. Inoculation protocols have been adapted specifically for studying different stages of infection – in general either early stages such as appressorium formation and epidermal infection or later stages such as arbuscule formation and root colonisation.

AM fungi belong to the order of Glomeromycota (Schussler *et al.* 2001). In contrast to the limited host specificity typical of rhizobial/legume associations, the AM fungal host range is very large and covers 80% of land plants. Several *Glomus* and *Gigaspora* AM species are commonly used in the laboratory: such as *G. intraradices*, *G. caledonium, G. versiforme*, and *Gi. rosea, Gi. margarita*, *Gi. gigantea.* The manipulation of AM fungi is not straightforward since these fungi are obligate biotrophs, and in general fungal inocula are prepared either from germinating spores or mycorrhizal roots.

The AM inoculation protocols described in this chapter have been divided into two categories, depending on whether **axenic** or **non-axenic** conditions are employed. Manipulating in **axenic** conditions requires the production of sterile fungal material and has the advantage of avoiding contamination with other pathogens or rhizobia. This approach is of particular interest since it is compatible with the use of Ri T-DNA transformed roots growing on sucrose-containing medium (Fortin *et al.* 2002). Sterile fungal material is either directly produced *in vitro* (spores or mycorrhizal roots, see Appendix) or obtained *via* sterilization of fungal spores produced in non-axenic conditions (see chapter Symbiotic interactions – Arbuscular Mycrorrhiza). Two "**axenic**" protocols are presented here (section **A**): the first of these is adapted for studying early stages of infection using Ri T-DNA transformed roots, and can be coupled with microscopy for *in vivo* dynamic analysis at the cellular level; the second has been specifically developed for gene expression studies in composite plants and is better adapted for "late stage" observations.

N**on-axenic** inoculation (section **B**), which can be used for well-developed plants, is appropriate for high-throughput experiments such as genetic screens and may be of particular interest for the large-scale production of mycorrhizal roots (e.g. for protein extraction). These conditions can be used for either seedlings or composite plants and are not suitable for early infection stage observations.

In the last section (**C**) we present various staining techniques for visualising the fungus in inoculated and colonised root tissues. Finally, the Appendix to this chapter describes two techniques for the *in vitro* production of *Glomus*/*Gigaspora* spores.

A1. Targeted inoculation of *in vitro* Ri T-DNA-propagated roots with pre-germinated *Gigaspora* spores.

Developed by:

Mireille Chabaud[1], Guillaume Bécard[2] and David G. Barker[1]

[1]: Laboratoire des Interactions Plantes-Microorganismes (LIPM), CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, France

[2]: Signaux et Messages Cellulaires chez les Végétaux (SMCV), UMR 5546, CNRS-Université Paul Sabatier, BP 41617, 31326 Castanet-Tolosan Cedex, France.

See also Chabaud *et al*. (2002)

***Contact* :** mchabaud@toulouse.inra.fr

1 - Preparation of fast-growing root explants.

For optimal growth of the explants prior to inoculation, roots are cultured **vertically** on M medium (composition below) containing **0.5 %** Phytagel (Sigma, St Louis, MO, USA) in two steps. An initial pre-culture is set up with 3 cm-long explants (primary root apex with several laterals), 4 explants/plate, grown on square Petri dishes (12 x 12 cm) for 2-3 weeks. In this pre-culture, fast-growing explants have a "fish-bone" morphology suitable for targeted inoculation. All cultures (initial root cultures and inoculated root explants) are incubated at 25 °C in the dark.

2 – Pre-germination of axenic spores.

Sterile spores are pre-germinated as described in Appendix 1. *Gi. rosea* spores are gently inserted into the M medium in square Petri dishes (9 x 9 cm) containing 25 ml of M medium (**0.5 %** Phytagel) and cultured at 32 °C at a slope of approximately 70° with a 2% CO_2 atmosphere to optimise germination (according to Bécard *et al*,. 1992). This takes between 3-6 days. Because germ tubes of *Gigaspora* species have a negative geotropism, once they reach the surface of the gel, they then grow along the Phytagel surface towards the top of the dish.

Sterile *Gi. gigantea* spores or *Gi. margarita* can also be used in this way (see chapter "Symbiotic interactions – Arbuscular mycorrhiza" for spore production and sterilization).

3 - Targeted inoculation of *Medicago truncatula* Ri T-DNA transformed roots.

Fresh germinated spores (germinating hyphae are about 0.1-0.5 cm long), are transferred in a gel plug to another square Petri dish of the same dimensions (9 x 9 cm) containing 25 ml M medium (**0.5 %** Phytagel) and a *M. truncatula* Ri T-DNA-transformed root explant, and placed just below a lateral root. *Note that it is essential that the identical concentration and thickness of medium is used for spore germination and root culture to facilitate transfer and subsequent hyphal growth.*

This approach allows observations of hyphal growth and fungal/root contacts using the binocular microscope. Hyphal branching and first appressoria are generally observed within 4-5 days of co-culture and arbuscules approximately one day later.

Remark:

This technique allows high-resolution microscopic observations of the infection process if the inoculated explant is first covered with a gas permeable plastic film (bioFOLIE 25; Sartorius

AG) and microscopy performed with long-distance water-immersion objectives (see Genre et al., 2005). In this case Gi. gigantea *is preferable to* Gi. rosea *because of its cytoplasmic autofluorescence.*

A2. *Glomus intraradices* inoculation of *in vitro* grown composite plants

Developed by:

Andry Andriankaja, Mireille Chabaud and Fernanda de Carvalho-Niebel.

Laboratoire des Interactions Plantes-Microorganismes, CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, France

Contact **:** fniebel@toulouse.inra.fr

A method has been developed which is well-suited for the rapid analysis of a large number of independent transgenic roots of composite plants generated by *A. rhizogenes* transformation. Approx. 3 weeks after *A. rhizogenes* inoculation (see chapter "Hairy root transformation with *Agrobacterium rhizogenes*"), composite plants with transformed roots selected on the basis of Km-resistance and/or constitutive expression of the fluorescent marker protein DsRED (Limpens *et al*. 2004) can be used for AM inoculation tests.

1 - Preparation of composite plants for AM inoculation

Composite plants with transgenic roots are transferred to a pouch-paper support laid on M medium (**0,5 %** Phytagel), **without sucrose** and without antibiotic selection in square Petri dishes (12 x 12 cm). *Note that in these antibiotic-free conditions, non-transformed roots that stopped growing in antibiotic-containing media will now reinitiate growth. Thus it is important to remove these non-transformed roots before transferring composite plants to the antibiotic-free paper support.*
3-4 composite plants are transferred per plate and immediately inoculated with a suspension of *G. intraradices* inoculum prepared as described below. About 100 spores are used per plate. Transgenic roots cultivated in the presence of spores are analysed 3-4 weeks after inoculation.

2 - Preparation of *G. intraradices* inoculum

In vitro-cultured *G. intraradices*-colonised carrot root cultures (with hyphae and extraradicular spores) cultivated on 90 mm plates with M medium (including sucrose) are used for inoculum preparation (see Appendix 2). Several month-old cultures (together with the culture media) from 1 or 2 plates are collected and gently mixed under sterile conditions using 30 ml of sterile water in a blender (about 3-5 seconds at low speed). This *G. intraradices* suspension, which contains about 25-30 spores/ml and colonised root fragments is directly used for inoculation of composite plant roots, applying 3-5 ml/ plate (approx 100 spores).

Remarks:

This method is also well suited for the inoculation of young seedlings. In this case, germinated seedlings are directly transferred to pouch-paper/M media ***without sucrose*** *and grown for 4-5 days before spore inoculation. Using this technique we have found that 100 % of analysed roots are colonised 3-4 weeks after inoculation with an average of 6 infection sites/root (particularly for lateral roots).*

M Medium composition: (from Bécard and Fortin, 1988)

Macro elements 20X	**Stock solution in 500 ml**	**Final concentration mM**
$MgSO_4$ $7H_2O$	7.31 g	3.0
KNO_3	0.8 g	0.79
KCl	0.65 g	0.87
Ca $(NO_3)_2$ $4H_2O$	2.88 g	1.22
Micro elements 100X		**µM**
KH_2PO_4	240 mg	35.0
Na Fe EDTA	400 mg	21.7
KI	37.5 mg	4.5
$MnCl_2$ $4H_2O$	300 mg	30.3
$ZnSO_4$ $7H_2O$	132.5 mg	9.2
H_3BO_3	75 mg	24.0
$CuSO_4$ $5H_2O$	6.5 mg	0.5
Na_2MoO_4 $2H_2O$	0.12 mg or 120 µl of solution 10mg/ 10ml.	0.01
Vitamins 100X		**µM**
glycin	150 mg	40
Thiamin HCl	5 mg	0.3
Pyridoxin HCl	5 mg	0.5
Nicotinic Acid	25 mg	4
Myo-inositol	2.5 g	277
Sucrose		10 **g/L**
Phytagel		3 to 5 **g*/L**
pH		5.5

NOTES:
- When entire plants are grown on M medium, sucrose is not necessary and hence should not be included.
- Because of the use of Phytagel as a gelling agent, M medium has to be poured immediately after autoclaving since Phytagel medium cannot be re-melted.
- A few drops of KOH (0.1M) is used to adjust the pH.
*: Note that 5 g/L Phytagel (instead of 3 g/L) is used for vertical culture.

For 1 L of M medium:
Use 50 ml Macro-element stock solution (20X), 10 ml of micro-element stock solution (100X), 10 ml of vitamin stock solution, adjust to 1 litre with ultrapure water, add 10 g of sucrose and adjust pH to 5.5 with KOH 0.1M. Then pour into a bottle containing 3 or 5 g/L Phytagel for autoclaving.

B - Inoculation in pots under non-axenic conditions:

Developed by:

Maria Harrison.
Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY, 14850

Summary
In this procedure, *M. truncatula* seedlings are grown until they have one trifoliate leaf. They are then transplanted into turface or sand and inoculated with arbuscular mycorrhizal fungal spores. The symbiosis will develop over the following 4 weeks.
This protocol has been used to colonize *M. truncatula* with *G. intraradices, G. versiforme, G. caledonium* and *Gi. rosea*.
To ensure that the symbiosis develops under optimal conditions, we routinely sterilize or surface sterilize all components.

1- Preparation of growth materials and pots

Items required:

- Chlorox (bleach)
- Fine sand (autoclaved)
- Coarse sand or Turface (autoclaved). Turface is a trade name for calcined clay.
- Plant pots (11 cm-diameter plastic pots)

Procedure:

a. The cones are surfaced sterilized before use. Soak cones in 10% chlorox for about 15 min. Rinse well by submerging in deionised water. Repeat the rinse at least 3 x and leave to dry. Treated cones can be stored in a clean plastic bag until needed.
b. The sand and turface are rinsed at least 3 x with double-distilled water and autoclaved before use.

2-Preparation of *M. truncatula* seedlings

Procedure:

a. For seed sterilization and germination, see chapter “Seed storage and germination”. Remove the seedlings from the cold and leave at room temperature for 2 days. Then remove the foil and leave at room temperature for an additional day. The seedlings will have germinated and will turn green after exposure to light.
b. Plant the germinated seedlings into sand or turface and allow them to grow until they have the first trifoliate leaf. During this time, they should be watered with a low phosphate fertilizer such as half-strength Hoaglands solution with 20µM phosphate.

3-Surface sterilisation of fungal spores

The fungal spores should be sterilised just before use. There are many protocols for surface sterilisation. We use the following method.

Items required:

- AM fungal spores (eg. *G. intraradices*)
- Chloramine T/ Tween 20 solution (Dissolve 0.4g Chloramine T in 20ml H_2O. Add 50µl Tween 20)
- Streptomycin sulphate (200ug/ml – Filter sterilized)
- Sterile distilled water

Procedure:

a. Place 200 spores in 20mL of fresh Chloramine T/Tween 20 solution. Stir gently for 15 minutes. Allow spores to settle then remove the solution. Repeat. Allow spores to settle and remove the solution.
b. Add 20 mL of streptomycin sulphate. Incubate at 4°C for at least one hour- the spores may be left in streptomycin overnight. Do not leave the spores in streptomycin for longer than one day as it may reduce germination.
c. Remove streptomycin and rinse five times with sterile distilled water. This is easily performed by filling the tube with water, allowing the spores to settle to the bottom, then removing water with a pipette.
d. Save the water from the last wash. It can be used for the mock-inoculation of the control plants.

4-Inoculating *M. truncatula* plants with AM fungal spores

Items required:

-*M. truncatula* plants (first trifoliate leaf stage)

-Sterilised sand, turface and pots

-Sterilised spores

Procedure:

a. Fill 2 plant pots approximately 2/3 full with sterile turface or coarse sand. Then add a 1cm layer of fine sand. Place 10 seedlings in each pot, spreading the roots out onto the fine sand. One pot will be inoculated with spores. The other pot will be a control and will receive the last spore wash.
b. Pipette 200 spores onto the roots of the plants in one of the pots. Cover the roots with turface or coarse sand. Pipette an equivalent volume of spore washing water onto the control plants in the second pot. Place both pots in a growth chamber (conditions: 16h/8h, 25°C, light intensity 200-230 µE). It is useful to cover the plants with a dome or plastic bag until they recover from transplanting.
c. Fertilize as needed (usually 1-2 x per week) with a low phosphate fertilizer. The symbiosis should be well developed within 4 weeks.

To harvest the roots, invert the pots and shake gently. The contents will fall out. Place the turface and roots in a large volume of water and shake gently. The turface will fall off the roots. The root can then be stained to enable observation of the mycorrhizal fungus.

C1. Ink staining of AM fungal hyphae.

Presented by: Mireille Chabaud.
Laboratoire des Interactions Plantes-Microorganismes, CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, France

(modified from Vierheilig *et al*., 1998)

Staining is performed on root fragments several cm in length. Each fragment is placed in an Eppendorf tube (with a hole in the cap made with a syringe needle to release air pressure in the tube during heating).

Root treatment:

a. 7 minutes in 10% KOH at 95°C. For very fine roots (produced *in vitro* for example), 6 minutes should be sufficient. On the other hand thick roots will require a longer treatment.

b. 3 rinses in water. Since roots are very fragile because of the treatment in KOH at high temperature, the rinses are made with a pasteur pipette trying not to touch the roots.

Staining:

a. 3 minutes in 5% black ink ("Noir de Jais" manufactured by Shaeffer) diluted in vinegar alcohol 8° (found in the grocery store) at 95°C.

b. 2 rinses in water with 10% vinegar. Same caution as before not to damage the roots.

Destaining:

Leave 20 minutes in the second rinse

Observations :

Roots must be observed the same day as the staining is not stable.

Note: it is possible to first stain roots histochemically for beta-glucuronidase activity with X-Gluc, and then rinse in water.

C2. Trypan blue staining.

Presented by: Maria Harrison.
Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY, 14850

Trypan blue is a suitable stain for a wide range of AM fungi.

Stock solutions

- 10% (w/v) Potassium hydroxide
- 2% (w/v) Trypan blue
- Lactoglycerol solution (combine 300ml Lactic acid, 300ml Glycerol, 400ml double-distilled water)

Procedure

a. Submerge the roots in KOH (10%) and heat at 90°C for 20 min.

b. Decant the KOH and rinse the roots twice with deionized water.

c. Prepare Trypan blue staining solution by mixing 25ml of Trypan blue stock and 1000ml Lactoglycerol

d. Cover roots in Trypan blue staining solution and place at 90C for 3-5 mins (DO NOT LEAVE THEM LONGER or they will turn completely blue)

e. Decant the stain into a waste bottle and place the stained roots in glycerol.

f. Mount roots in glycerol on slides for microscopy (NOTE – Do **not mount** in lactoglycerol, it destroys the microscope!). The fungus will be stained blue and should be clearly visible within the roots. If the fungus has not stained enough, repeat the staining step. If the roots have stained too much, place them in lactoglycerol and they will destain.

g. The roots from the mock-inoculated controls should be stained and examined. These serve as a control and will indicate the quality of the growth conditions. Obviously they should not contain any mycorrhizal fungi.

C3. Acid fuchsin staining.

Presented by: Maria Harrison.
Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY, 14850

Stock Solutions:

- 500 ml 50% ethanol (95% ethanol is under the hood. Dilute to 50% with distilled water)
- 0.1 M HCl (stock solution under the hood)
- 20% potassium hydroxide (KOH) solution
- 50% glycerol
- Acid fuchsin staining solution contains:

 140 ml lactic acid (if lactic acid stock solution is 85%)
 10 ml glycerol
 10 ml distilled water
 0.01 % acid fuchsin

 Prepare an acid fuchsin stock solution (e.g. 25% in water) to avoid breathing the dust of this compound (work with a mask or in the hood). The acid fuchsin staining solution and stock solution both last a very long time at room temperature

Rinsing solution: Same as the staining solution but without the acid fuchsin.

Staining procedure:

a. Harvest roots and wash gently in distilled water to remove turface and/or sand.

b. Place roots in 50% ethanol (enough to cover the roots) and leave for at least 1 hour. Overnight or longer is fine.

c. Remove ethanol. Add 20 % KOH (enough to cover the roots) and leave for 2-3 days.
d. Remove potassium hydroxide and rinse with distilled water.
e. Add 0.1M HCl (enough to cover the roots) and leave for 1-2 hours.
f. Remove acid and leave at least 1 day in the staining solution (enough to cover the roots).
g. Transfer into the rinsing solution and then into 50% glycerol. Cut into pieces approx 2cm long and place on microscope slides for observation.
h. (If stored in glycerol roots will destain).

Do not put the lactic acid/glycerol mix on the microscopes, it is corrosive.

NOTE: Acid fuchsin stains the fungus pink when viewed by light microscopy. It is also fluorescent and the fungus will appear orange/yellow using an epifluorescence microscope. Use a filter set for rhodamine/Cy3 or TRITC for observation.

Appendix

Presented by:

Mireille Chabaud[1], Nadja Feddermann Bühler[2], Guillaume Bécard[3]

[1]Laboratoire des Interactions Plantes-Microorganismes, CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, France

[2] Botanisches Institut der Universitt Basel Labor Nr. 110 Hebelstrasse 14054 Basel (Switzerland)

[3]Signaux et Messages Cellulaires chez les Végétaux, UMR 5546, CNRS-Université Paul Sabatier, BP 41617, 31326 Castanet Tolosan Cedex, France.

Contact: mchabaud@toulouse.inra.fr

Here we present two techniques as examples for *in vitro* spore production.. The first was developed for *Gigaspora rosea* using *M. truncatula* hairy roots and the second for *Glomus intraradices* using carrot hairy roots. However, *Gi. rosea* spores can also be produced with carrot roots and *vice versa* for *G. intraradices*.

Appendix 1 – *In vitro* production of *Gigaspora rosea* spores.

This protocol is derived from Diop *et al.* (1992). Sterile spore production requires 2 to 3 months of culture. On average, 4 pre-germinated spores produce 50-60 new spores (a multiplication factor of around 12-15 fold), but production is highly variable from one plate to another (from 1 to 190 newly formed spores/plate).

1 - Preparation of the root explants.

The procedure for obtaining Ri T-DNA transformed roots of *M. truncatula*, their bacterial decontamination and subsequent root propagation is described in Boisson-Dernier *et al.* (2001) and in chapter “Hairy root transformation with *Agrobacterium rhizogene*s ». Root clones are propagated by subculture every 4 weeks in large horizontal Petri dishes (diameter= 14 cm) containing M Medium (composition below) with **0.3 %** Phytagel (Sigma, St Louis, MO, USA). Culture is performed in the dark at 25 °C.

2 – Pre-germination of axenic spores of *G. rosea* Becker and Hall (DAOM 194757).

Sterile *G. rosea* spores are gently inserted into M medium containing **0.5 %** Phytagel in square Petri dishes (12 X 12 cm) and cultured at 32 °C at a slope of approximately 70° with a 2% CO_2 atmosphere (according to Bécard *et al*,. 1992). Within 3-6 days germinating hyphae appear and grow upwards because of negative geotropism. The use of synchronised pre-germinated spores is optimal for mycorrhization and reduces the overall time of the culture.

3 - Inoculation of *Medicago truncatula* Ri T-DNA transformed roots and spore production.

Healthy-growing root explants from 15-20 day-old cultures are used for inoculation. A single explant (about 4 cm long, comprising the primary root with several laterals) is placed in the bottom part of a square Petri dish (12 x 12 cm) containing M medium (**0.5 %** Phytagel). Germinated spores (with hyphae about 0.1-0.5 cm long) are transferred in a gel plug to the Petri dish containing the explant and placed just below a lateral root. 4-5 pre-germinated spores are used per dish containing the single explant. Petri dishes with their inoculated explant are placed vertically in the culture room. Transformed roots grow down to the bottom

of the dish (positive geotropism) and the fungus develops in the upper part of the plate (Figure 1).

The first new extraradicular spores appear within 4-6 weeks and maximal production is reached within 2 to 3 months.

4 - Spore conservation and germination of newly-formed spores.

Once maximal spore production has been reached dishes are placed horizontally upside down (to avoid contact between water condensation and the spores) at 4°C for conservation. Several weeks of cold treatment synchronizes germination of newly produced spores. Following 2-4 weeks at 4°C, germination is around 80%. Spores can then be kept at 4°C for several months but the germination efficiency tends to decrease with time. Therefore, spore production must be repeated on a regular basis (every 1-2 months) in order to have young spores with high germination rates always available.

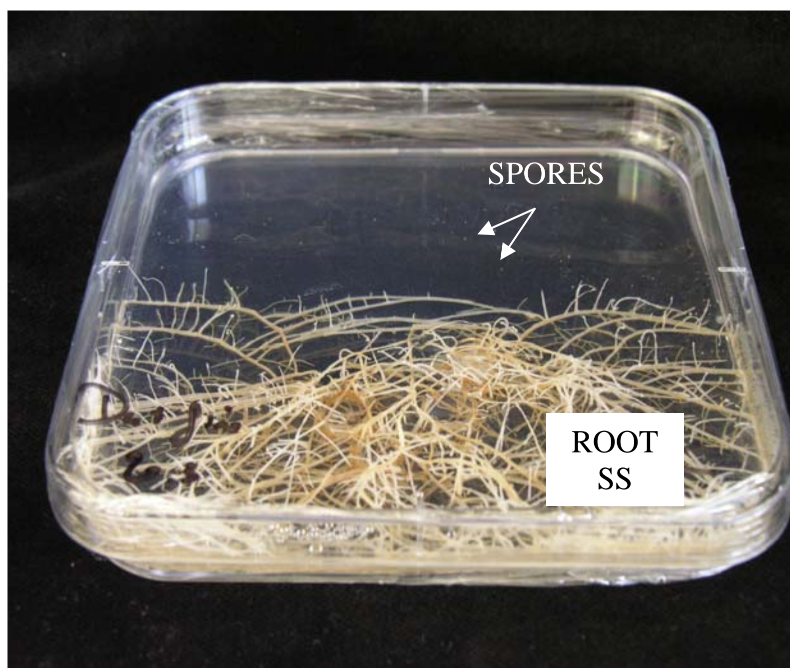

Figure 1: *In vitro* production of *Gi. rosea* spores using an Ri T-DNA transformed *M. truncatula* root culture.

Appendix 2 – *In vitro* production of *Glomus intraradices* spores

1 - Inoculum material

Axenic *Daucus carota* Ri T-DNA root cultures, colonised by *G. intraradices* are grown in Petri dishes (diameter: 10 cm) containing M medium (see composition below) with **0.3 %** Phytagel for 5 weeks at 25 °C in the dark.

2 - Sub-culture of mycorrhized roots

Every 5 weeks, several colonised carrot root segments (2-3 cm long) are transferred to a fresh Petri dish containing M medium. Additional extraradicular spores of *G. intraradices*, which have developed in the medium of the inoculum plate, are placed on the root pieces.
This regular sub-culturing ensures satisfactory growth of the AM-colonised roots.

3 – Spore collection and isolation

With a scalpel blade the spore-containing medium is removed from the plate, and placed in an Eppendorf tube. The medium is dissolved in citric acid (50 mM). Spores are rinsed with distilled water and collected from the bottom of the tube.
The medium plug can also be chopped into pieces and used directly for inoculation of roots.

Reference: Fortin *et al.* 2002.

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