Experiment: Phytoplasma isolation

Date of experiment: September 2017
Analyst: Dorian Dolanc
Observer: Nataša Mehle

Laboratory material:

- scalpel
- small forceps
- bunsen
- sterile plastic dropper
- sterilized filter paper
- Petri dishes, polystyrene, size 60 mm x 15 mm (Sigma, P5481-500EA)
- V-Monovette Urine 10 mL Round base (Marketlab, ML13814)
- 0.8 µm syringe filter non pyrogenic hydrophilic (Sartorius Stedium, 16592 GUK)
- 0.22 µm filter (Sartorius Stedium, 16532 GUK)
- autoclave
- anaerobic jar (Oxoid)
- ddH_2O

Chemicals:

- 1% NaClO solution - Sodium hypochlorite solution (Sigma, 1.056.142.500)
- ethanol (for surface sterilization)
- CampyGen sachets (Oxoid, CN0025; for microaerophylic athmosphere production)
- TSB (Oxoid, CM1065) (TSB is a multipurpose medium containing essentially tryptone and soya peptone, and supporting the growth of a wide range of bacteria)
- MBB (Oxoid, CM0403) (Mycoplasma Broth Base is a basic medium containing bacteriological peptone and lab-Lemco powder used in isolation and cultivation of mycoplasmas).
- horse serum (Oxoid, SR0035)
- ampicillin (Sigma, A9393)
- nystatin (Sigma, N6261)
- yeast extract (Sigma, Y1625-250G)
- phenol red (Sigma, 1.072.410.005)
- agar No.3 (Oxoid, LP0013)
- NaCl

Samples:

Leaves of grapevine:
D667/17: BN positive, FD negative
D668/17: phytoplasma negative
D669/17: FD positive, BN negative
D670/17: phytoplasma negative

CBI or MBI medium

<u>CBI medium:</u>
Add TSB powder into ddH_2O to reach concentration of 30g/l (pH 7.3 +/- 0.2).
After 20 min sterilization at 121ºC in autoclave, the media has to be enriched with the supplement listed below.
Into each 80 ml of medium add:

- 20 ml of sterile horse serum,
- 25 µg/ml of ampicillin (0.22 µm filter sterilized)

- 50 µg/ml of nystatin (0.22 µm filter sterilized)
- 10 ml of autoclaved yeast extract (25% w/v)
- 0.005% of phenol red

MBI medium:
Add MBB powder into ddH_2O to reach concentration of 25.5 g/l (pH 7.8 +/- 0.2).
After 20 min sterilization at 121ºC in autoclave, the media has to be enriched with a supplement listed below. Into each 80 ml of medium add:
- 20 ml of sterile horse serum,
- 25 µg/ml of ampicillin (0.22 µm filter sterilized)
- 50 µg/ml of nystatin (0.22 µm filter sterilized)
- 10 ml of autoclaved yeast extract (25% w/v)
- 0.005% of phenol red

Solid CBI medium:
Add TSB powder into ddH_2O to reach concentration of 30 g/l (pH 7.3 +/- 0.2). Then add:
- 20 g/l of NaCl
- 12 g/l of agar No.3
Autoclave it, then add:
- 25 µg/ml of ampicillin (0.22 µm filter sterilized)
- 50 µg/ml of nystatin (0.22 µm filter sterilized)

Solid MBI medium:
Add MBB powder into ddH_2O to reach concentration of 25.5 g/l (pH 7.8 +/- 0.2).
- 20 g/l of NaCl
- 12 g/l of agar No.3
Autoclave it, then add:
- 25 µg/ml of ampicillin (0.22 µm filter sterilized)
- 50 µg/ml of nystatin (0.22 µm filter sterilized)

PROTOCOL

1. Extract the midribs from the leaves using a scalpel

Figure 1. Midribs from grapevine (*Vitis vinifera*).

2. Surface sterilization of midribs from the leaves: immerse the midribs in hypochloride (1% NaClO) for 1 minute, rinse them twice in two separate ddH_2O containers and dry them using a sterilized filter paper.

Figure 2. Surface sterilization of the midribs.

Figure 3. Drying the midribs.

Comment: we also tried two methods for surface sterilization of the midribs using ethanol. In the first method, instead of using NaClO and ddH_2O, we immersed the midribs into 98% ethanol and scorched, so the ethanol evaporated. The second method involved surface sterilization using NaClO and ddH_2O, after which the midribs were immersed into 98% ethanol and treated as in the first method.

3. On both ends of the midrib cut a little part off and discard it. Pour the liquid media on a sterile plate and immerse the midrib pieces. Cut the immersed midribs in little pieces, as small as possible. Make sure you squeeze the midrib pieces as you cut them with the scalpel, to extract the juice and present inside the midribs.

Figure 4. Immersing the midrib piece.

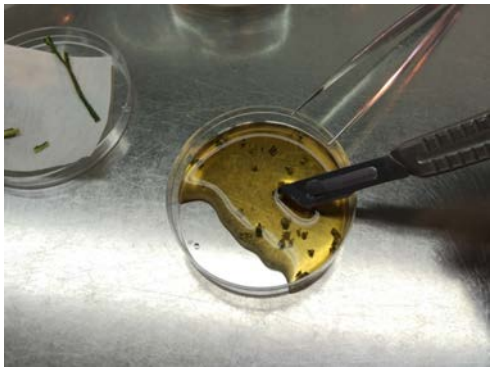
Figure 5. Cutting the midrib in pieces

4. Transfer medium and midrib pieces into a monovette tube using a sterile monouse plastic pipette. Make sure you do not pick up all the midrib pieces. A few pieces should be enough.

(comment the medium should be orange not yellow at this stage!)

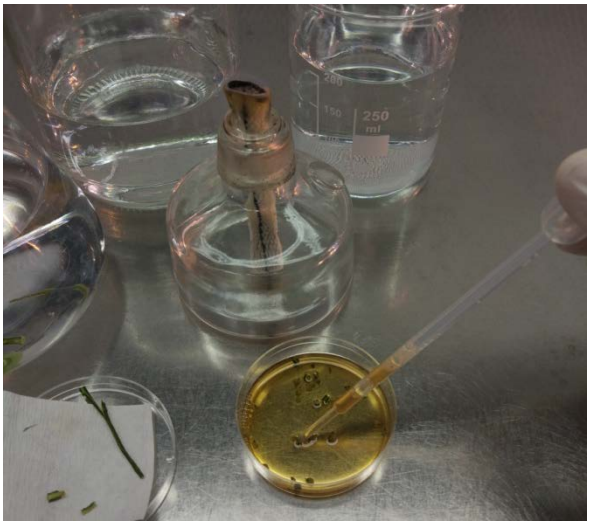

Figure 6. Picking up liquid media and the midrib pieces.

Figure 7. Monovette tube containing liquid media and midribs

5. Incubate the inoculated liquid media at 25 +/- 1 ºC under normal atmospheric conditions and in dark.

6. Media contains phenol red which in the case of acidic pH turns from orange-red to an orange-yellow colour (because of metabolic activity in the media). Make dilutions from the tubes that change colour. Make sure you transfer the liquid from the middle of the tube without mixing the media. Transfer 3 drops (about 100 ul) to a tube containing fresh medium and two drops (about 60 ul) into a plate with solid medium. Spread the two drops with a 10 ul loop.

Figure 8. Transferring the inoculated medium.

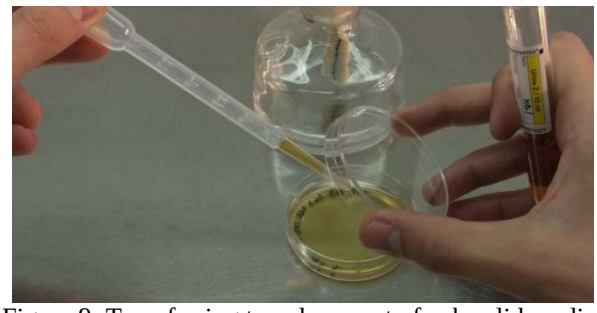

Figure 9: Transferring two drops onto fresh solid media.

7. Incubate the inoculated liquid media at 25 +/- 1 ºC under normal atmospheric conditions and in dark. The inoculated solid media plates must be incubated at the same conditions in microaerophilic athmosphere. For that, use an anaerobic jar and CampyGen sachets. For negative controls add tetracycline 25 µg/ml to the media and perform the above described isolation procedure using midribs from healthy grapevine.

8. Results: in our study only endophytic bacteria have been seen on solid media (Figure 10). We did not get any phytoplasma colony as published by Contaldo *et al*., 2016 – see their figure (Figure 11). No timing is provided, colonies are only visible under optical microscope in the first days after plating and this was not performed.

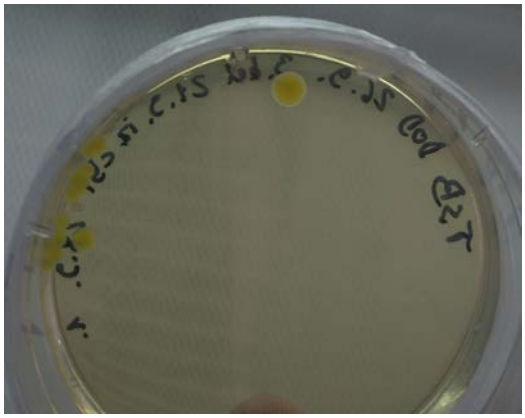

Figure 10. Endophytic bacteria colonies.

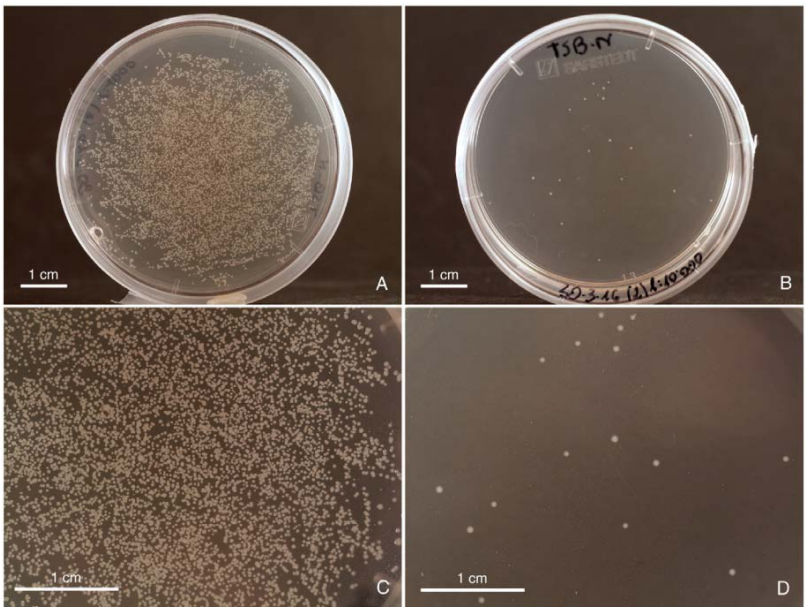

Figure 11. Phytoplasma colonies (Contaldo *et al*., 2016)

REFERENCE

Contaldo N., Satta E., Zambon Y., Paltrinieri S., Bertaccini A. 2016. Development and evaluation of different complex media for phytoplasma isolation and growth. Journal of Microbiological Methods, 127: 105-110.