SOP Isolation of legume root nodule bacteria

Protocol first designed and optimized by Sofie De Meyer with help of Bram Vekeman, Tamara Braeckman (De Meyer et al. 2011).
Revised and updated (October 2013) by Liesbeth Lebbe and Anne Willems.

1. Method

1.1. Preservation nodules

The nodules from the root system of each plant are removed and can be stored in plastic screw-capped tubes filled with silica beads. Place the nodules on some cotton-wool above the silica. The moisture-absorbing crystals ensure that the nodules will be dehydrated and can be preserved for several months at 4°C.

1.2. Rehydration

Soak the nodules (e.g. 3 per plant) for at least 30 minutes in a glass tube with sterile distilled water (AD). They will rehydrate and should take on their original shape.

1.3. Surface sterilization

After rehydration, the nodules are separately subjected to a surface sterilization to remove the bacterial flora on the outside of the nodule.
1. Bring the nodule in 300µl sterile AD in eppendorf tubes + 0.12g sterile glass beads (ø 0.5mm), vortex 30-60 sec. Thereby, large impurities such as sand and soil residues are dislodged.
2.Transfer to fresh eppendorf tube containing 1ml sodium hypochlorite solution 3% and leave for 3 min while mixing (vortex) several times. The nodules are always transferred from one eppendorf to another with sterile forceps. Be careful not to damage the nodule while handling.
3. Transfer to a fresh eppendorf tube with 1ml of sterile AD plus 0.12g sterile glass beads (ø 0.5mm), vortex 30-60 sec.
4. Transfer to a fresh eppendorf tube with 1ml of sterile AD, vortex 30-60 sec and repeat this wash step three more times so that the nodule has been washed in total 5 times (once with beads present and 4 times in just sterile AD).

1.4. Check the surface sterilization process

After the surface sterilization process, the nodules are being rolled over a petridish with YMA (Yeast Mannitol Agar) to verify that all external bacteria were removed. Most rhizobia and other bacteria that might otherwise contaminate subsequent isolations will grow on YMA. If the surface of the nodule was completely disinfected, there should be no more growth upon incubation of this plate at 28°C for 2-3 days. If growth does develop, the nodule is not used further as any isolates from it may not be truly internal bacteria.

1.5. Isolation

Squash the nodule in 300µl sterile physiological water (0.86% NaCl) using a sterile glass rod. For the isolation of rhizobia, 50µl of a dilution series of this suspension is plated on YMA (dilutions 0x-4x-10x-20x for small nodules, 10x-50x-100x for medium nodules, 1000x for large nodules). Incubate plates of the dilutions at 28°C for up to 20 days. The purpose of the dilutions is to prevent that slow-growing bacteria are overgrown by fast growers.
Growth should be checked regularly. Approximately 3 different colonies can be selected by use of a stereo microscope. Working aseptically in a biosafety cabinet, pick up the selected colonies with a sterile öse to subculture them on a new YMA plate for further purification to pure cultures. The pure cultures can be stored frozen in cryovials (Microbank) or in YM broth + 15% glycerol.

2. Materials

2.1. Sterile AD

Fill some bottles (0.5 or 1 l) with AD and small glass (5 ml) vials with 1 ml AD and sterilize.

2.2. NaClO 3%

Sterilize a bottle with 78.57ml AD. Add afterwards 21.43ml NaClO 14% aseptically. Store shielded from light (dark bottle).

2.3. Sterile glass beads and glass rods

Sterilize glass beads and rods in a drying oven. (6h-96°C)

2.4. Physiological water

Prepare a solution of 0.86% NaCl and sterilize.

2.5. YMA (Yeast Mannitol Agar, Vincent 1970), BCCM/LMG M203

Mannitol	10 g
K2HPO4	0,5 g
Sodium glutamate	0,5 g
NaCl	50 mg
Solution A	10 ml
Solution B	1 ml
Solution C	1 ml
Yeast extract	1 g
Agar	20 g

Distilled water up to 1 L, pH 6.8

Solution A:
MgSO4 x 7H2O 1 g
Distilled water 100 ml

Solution B:
CaCl2 x 2H2O 5,28 g
Distilled water 100 ml

Solution C:

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FeCl3 x 6H2O 666 mg
Distilled water 100 ml

3. References

De Meyer S.E., B. Vekeman, T. Braeckman, K. Van Hoorde and A. Willems. 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol. Bioch. **43:**2384-2396. (doi:10.1016/j.soilbio.2011.08.005).

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