MICROBES IN THE ENVIRONMENT — Isolation of *Rhizobium*, a nitrogen-fixing bacterium

Rhizobium occurs both in a free-living form in soil and in the root nodules of legumes in a symbiotic association with the host plant. Nitrogen fixation occurs only in the root nodules, not when the bacteria are free-living. In the investigation, the outside of the nodule must be chemically sterilised and the nodules crushed to release the *Rhizobium.* The extract is inoculated onto an agar culture medium that contains sources of fixed nitrogen (in this case yeast extract), carbon, energy (mannitol) and some mineral salts. The inability of rhizobia to fix nitrogen when in the free-living form may be demonstrated by investigating their ability to grow on a nitrogen free medium.

Learning Objectives

To show:

- the role of microbes in maintaining the nitrogen cycle
- how microbes may be isolated from root nodules
- an example of symbiosis

Age Range

Year 10 and above

Duration

Session 1: 60 minutes
Session 2: 20 minutes
Incubation period: 25ºC for 3-4 days

Materials (each group)

legume plants (clover/pea/bean) with root nodules
Bunsen burner
wire loop in holder
scalpel
forceps, metal, fairly fine
marker pen
adhesive tape
beaker of disinfectant
70% (v/v) industrial methylated spirits in a small beaker covered in foil (CAUTION: flammable, should be kept covered away from lit Bunsen)
incubator at 25°C
mannitol-yeast extract agar (MYEA)* plate
mannitol agar (MA)** plate
5 sterile Petri dishes
sterile distilled/demineralised water
sterile Pasteur (dropping) pipettes, plugged
sterile glass rod, wrapped

***Mannitol-Yeast Extract Agar (MYEA) (g/l)**

10.0g	mannitol	1.0 g	yeast extract
0.5g	K_2HPO_4	0.2g	$MgSO_4.7H_2O$
0.1g	NaCl		

Dissolve ingredients in 1 litre water, adjust to pH 7.0.
Add solution to vessels containing
0.3g (pro rata) $CaCO_3$
15.0g (pro rata) agar
Sterilize by autoclaving. Distribute $CaCO_3$ evenly before pouring plates.

****Mannitol agar (MA)**

As for MYEA but without yeast extract.

Notes

1. Advance planning is necessary to provide suitable plant material. Clover (seeds obtainable from school science suppliers) is recommended, as the nodules are relatively soft.

2. As an alternative to the mannitol yeast extract agar, use potato dextrose agar supplemented with 0.25g per litre of yeast extract.

3. The colonies of *Rhizobium* are off-white and sticky in appearance. Colonies of other colours are not *Rhizobium*; they may either be intracellular contaminants from the nodule or soil microbes that have survived the washing and disinfectant treatment.

Questions

Session 1

1. What is the purpose of the disinfectant treatment?
2. What kinds of plants have root nodules?
3. What is in the root nodules?
4. What is the name given to the relationship between the bacteria and the plant?
5. How does this relationship help the plant and does it help the bacteria?
6. Why use MYEA plates and MA plates?
7. What do you expect to see growing on your agar plates next session?

Session 2

8. Describe the appearance of the MYEA plates.
9. Is there one type of microbe growing or more than one?
10. Where have most of the microbes come from?
11. Where might the other microbes have come from?
12. How would you obtain a pure culture of the nitrogen-fixing bacteria?
13. Examine the MA plates for growth, explain the outcome.

MICROBES IN THE ENVIRONMENT

Isolation of *Rhizobium*, a nitrogen-fixing bacterium

Leguminous plants such as peas and beans usually have lumps or nodules on their roots. A bacterium called *Rhizobium* grows within these nodules which is able to fix, that is reduce nitrogen gas (combined with hydrogen) to ammonia. Green plants, the main producers of organic matter, use this supply of fixed nitrogen to make proteins that enter and pass through the food chain. You are going to isolate some of these microbes and grow them.

Procedure

Learning Objectives

To show:

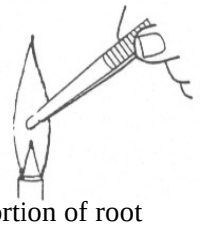
- the role of microbes in maintaining the nitrogen cycle
- how microbes may be isolated from root nodules
- an example of symbiosis

(1) Choose a length of root that has nodules and cut off a portion *ca* 1cm long using a scalpel. Hold the portion of root with forceps and wash free of soil using tap water.

(2) Transfer several drops of *70% (v/v) industrial methylated spirits* to a sterile Petri dish using a Pasteur (dropping) pipette fitted with a teat. The pipette need not be sterile for this operation. Place the pipette into the discard pot. Transfer the washed portion of root to the alcohol in the Petri dish with forceps and leave immersed for 1-2 min.

Use aseptic technique from this stage forward.

(3) Using a sterile Pasteur pipette fitted with a teat, transfer sufficient sterile water to cover the base of another Petri dish. If it is necessary to re-use the pipette, keep it sterile, e.g. by resting in a sterile Petri dish base and lid.



(4) Sterilise the forceps by dipping in alcohol and flaming off and use them to transfer the portion of root to the sterile water in the Petri dish in order to rinse off the alcohol. Repeat this operation at least twice more with fresh sterile water.

(5) Pipette a few drops of sterile water to another sterile Petri dish and add the portion of root using sterile metal forceps. Macerate the nodules using a sterile glass rod (or forceps) to produce a milky fluid. Discard the rod into the beaker of disinfectant.

(6) Sterilise a wire loop by flaming, cool it, take a loopful of the nodule macerate and streak it out on a labelled mannitol-yeast extract agar (MYEA) plate. Re-flame the loop. Prepare another streak plate on mannitol agar medium (MA), i.e. a N_2-free medium. Tape the plates and label the bases with your name. Invert the plates and incubate at 25°C for 3-4 days. Dispose of contaminated materials appropriately

Next lesson …

Examine the MYEA and MA plates without removing the lid, noting the appearance of any colonies growing on the agar. Answer the questions.

***Credits**: Thanks to John Grainger for practical information*

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