

Chapter 2.2

A PROCEDURE FOR *IN VITRO* NODULATION STUDIES

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*Standardised in vitro conditions yielding successful, synchronised, reproducible cycles of symbiotic nitrogen fixation are essential experimental tools for investigating the different stages of the Rhizobium-legume interaction. A standard in vitro procedure can facilitate analysis of the kinetics of phenotype at the molecular level occurring after Rhizobium infection. This is essential when comparing, for instance, the expression pattern of nodulin or marker genes or the phenotypes of wild type and mutant lines. We present here a feasible, reliable procedure for in vitro nodulation studies of *L. japonicus*. All the different steps of the symbiotic interaction have been analysed and different parameters of plant growth followed to demonstrate the reproducibility of the procedure.*

MATERIALS AND METHODS

Media

Bacterial medium (TyR)

- Bacto triptone 5 g/L
- Yeast extract 5 g/L

After sterilization, add CaCl₂ to a final concentration of 6 mM

Plant germination and growth medium (Jensen's)

To prepare 1 litre of 0.1% Jensen medium, use 1 ml of each of the below solutions:

- CaHPO₄ 40 g/400 ml
- K₂HPO₄ 8 g/400 ml

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- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 8 g/400 ml
- NaCl 8 g/400 ml
- Fe citrate* 4 g/400 ml

*Fe citrate can be added in appropriate amounts directly into the medium before autoclaving, without preparing a stock solution.

Add 2.5 μl of the below per 1 litre 0.1% Jensen medium:

- $\text{Na}_2\text{MoO}_4 \cdot 4\text{H}_2\text{O}$ 16 g

Add 2.5 μl of the below per 1 litre 0.1% Jensen medium:

- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.4 g/l
- $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 24.4 g/l
- $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 3.88 g/l

Add 25 μl of the below per 1 litre 0.1% Jensen medium:

- H_3BO_3 (10 x stock) 5.08 g

To solidify Jensen's medium, add 1% plant agar (Duchefa). Both TyR and Jensen's media are sterilized for 20 min. at 120°C.

Instrumentation

Preparation of Petri dishes

The Petri dishes used are 140x10 mm. After sterilization, 85 ml of 0.1% Jensen medium is poured into each Petri dish in a laminar flow cabinet. After drying for 20 min., one semicircle of filter paper is added in each Petri dish. Petri dishes, closed by Parafilm could be stored for 1-2 months at 4°C.

Filter paper preparation

Semicircles of filter paper with average diameter 5 cm are cut following the border of the base of the Petri dishes and wrapped in aluminium foil and sterilized for 30 min. at 120°C. Under sterile conditions in a laminar flow cabinet, each paper is put on the top of the already poured 0.1% Jensen's medium using forceps.

Growth conditions

Germination of the plants and their further growth after infection takes place at 23°C, with 16 /8 hours light/dark period and light intensity of 246 $\mu\text{E s}^{-1} \text{m}^{-2}$.

Procedure

Seed sterilization and germination

Lotus japonicus B-129 GIFU ecotype seeds are transferred to Eppendorf tubes (up to 100/Eppendorf tube), surface sterilized for 20 min in 25% commercial bleach (1%

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hypochlorite) and 0,1% Triton, washed 6 times in sterile H₂O and kept over night in water at 4°C. After 20-24 hours, the seeds are transferred to Petri dishes (140X10 mm), containing 0.1% solidified Jensen medium with a semicircles of sterilized filter paper placed on the top of the medium as to cover half of the Petri dish. Seeds are placed on the border of the filter paper (ten seeds for each Petri dish), so that the emerging roots will stay attached to it. The seeds are left overnight in the dark at 4°C lid side down before Petri dishes are moved into the culture cabinet. Here they are arranged in vertical position and kept for 24 hrs in the dark. Then the seeds are exposed to the light. At this step, it is important to maintain the young emerging roots in contact with the filter paper.

Preparation of the bacterial suspension

A single bacterial colony of *Rhizobium loti* R7A strain is grown overnight at 30°C in TyR medium, containing 6mM CaCl₂ and 100µg/ml Rifampicin. The day of inoculation, bacterial cells are collected by centrifugation at 4500 rpm for 10 min and rinsed twice with 1XPBS solution. The OD then is measured at λ=600 and 20 µl of bacterial suspension with 5.10⁷ cells are used for inoculation of each primary root meristem.

Infection

The inoculation is performed 4-5 days after germination of the seeds (primary root length about 1 cm). It is important to discard the unsynchronised seedling. Each primary root meristem is inoculated with 20 µl of the freshly prepared bacterial suspension and Petri dishes are sealed with Parafilm. The filter paper support is crucial to obtain a complete and uniform diffusion of the drop of bacteria over the root meristems. Four days after infection, the filter paper is removed (to avoid drying) and the plants are left for further growth on the same Petri dishes. This is done simply by taking the plants out of the Petri dish, removing the filter paper using forceps, and putting back the plants into the same Petri dish, paying attention to provide a direct contact of the roots with the medium. Aluminium foil is wrapped around the lower part of the Petri dishes to keep roots in the dark.

CONCLUSIONS

We screened the plants for different parameters of growth and symbiotic phenotypes five weeks after inoculation with *M loti* (Figure 1). The pattern of nodulation is uniform and the plants show conserved root and shoot phenotypes (Table 1). The basic experimental procedure reported here could be further improved by addition, into the Jensen medium, of factors improving the efficiency of nodulation as the ethylene production inhibitor, L-α-(2-aminoethoxyvinil) glycine (AVG) which is known to stimulate the nodulation of *L japonicus* (Pacios Bras et al., 2000, MPMI Vol.13: 475-479).



Figure 1. *L japonicus* plants five weeks after inoculation with *M loti* on 0.1% Jensen's medium.

However, the results in Table 1 show a detailed analysis and standard deviation of different parameters of nodule, root and shoot development that indicate a high reproducibility of the infection process. Such a reproducible experimental tool gives the opportunity for precise screening and detection of weak phenotypes.

| Parameters | Average mean (\pm SD) |
|-------------------------------|--------------------------|
| Number of nodules | 8 ± 2 |
| Primary root length | 7.2 ± 0.4 |
| Number of secondary roots | 5.2 ± 0.6 |
| Shoot length | 8.5 ± 1.2 |
| Number of trifoliolate leaves | 6 ± 1.3 |

Table 1. Average mean of different growth parameters calculated 5 weeks after inoculation with *M loti* on 0.1% Jensen medium. The results summarize the data from the screening of 900 plants.

REFERENCES

Pacios Bras C, Jorda MA, Wijfjes AH, Harteveld M, Stuurman N, Thomas-Oates JE, and Spaik HP. (2000) A *Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NoIL in *Rhizobium leguminosarum*. *Molecular Plant-Microbe Interactions*. 13, 475-479.