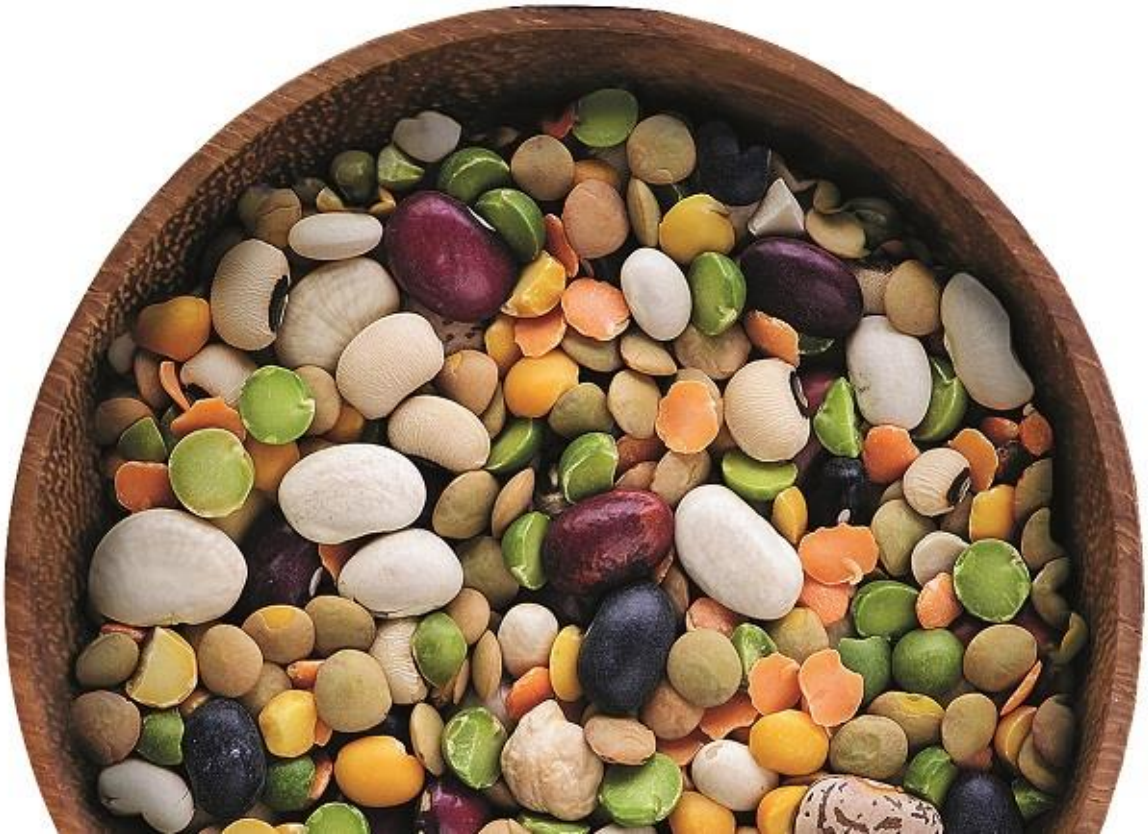
TRansition paths to sUstainable legume-based systems in Europe

Authentication of rhizobia (screening strains for nodulation)

Date: September 30th 2019



This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

Content

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

1 Summary Information

1.1 Partner Summary

SOP Code	EU_TRUE_SOP_048
TRUE Partner Acronym	AUA
Primary Author	Tampakaki, Anastasia P. (*tampakaki@aua.gr*)
Other Authors	Efstathiadou, Evdoxia
Linked Reference and Hyperlink (if available)	Somasegaran P. and Hoben H. 1994. Handbook for rhizobia: methods in Legume-rhizobium technology. Springer-Verlag: New York. https://www.springer.com/gp/book/9781461383772 doi: 10.1007/978-1-4613-8375-8 Vincent JM (1970) Appendix III. The modified Fåhraeus slide technique, In: Vincent JM, editor, A Manual for the Practical Study of Root Nodule Bacteria, Oxford: Blackwell Scientific, 144–145. https://doi.org/10.1002/jobm.19720120524
Associated files to use with the SOP [and function]	Not Applicable

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

1.2 SOP Summary

Title

Authentication of rhizobia (screening strains for nodulation)

Brief description

Authentication is the term used to obtaining proof that a given isolate is indeed the nodulating rhizobium. Ideally, a putative nodulating strain is tested for its ability to nodulate legume species from which it was originally isolated (Koch's postulates). Alternatively, the rhizobial strain could be tested on another legume species from the same cross-inoculation group, particularly when a small-seeded legume can be substituted for a large-seeded one. Large-seeded legumes like beans (*Phaseolus vulgaris*), Leonard jars and growth pouches are recommended as growth units for authentication, although beans with small seeds can be successfully grown in glass tubes (Somasegaran and Hoben, 1994).

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

2 Protocol Steps

2.1 Seed sterilization and germination

1. Put 10 seeds in a Petri dish (9-cm diameter) with tweezers or in a beaker or falcon tube.

2. Immerse the seeds in 1.5% (v/v) of commercial bleach for 15 minutes with constant mixing in a laminar flow hood then remove the bleach. Approximately add 10 seeds in 25 mL bleach or 10g seeds/20 mL.

3. Rinse the seeds with sterile distilled water at least three times over a 20 min period.

4. Germinate the sterilized seeds on sterile filter papers moistened with 7 mL sterile distilled water or sterile liquid medium within a petri dish for 2 days in the dark at 28 °C.

5. Keep the Petri dish the dark but place at RT.

6. After an incubation of 2 days, choose the germinated bean seeds with a root of 1-2 cm for inoculation.

2.2 Setting up the glass tubes containing plant culture medium

1. For each of the isolates, set up three growth units plus at least three extra units that will serve as uninoculated controls. An adequate bacteriological positive control should also be included in the test.

2. Prepare a nitrogen-free Fåhraeus plant nutritive solution (0.7 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 20 µM ferric citrate, 5 µM H_3BO_3, 10 µM $MnSO_4$, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4$, and 0.6 µM Na_2MoO_4), and adjust the pH to 6–7 (Vincent, 1970).

3. Add agar powder at 6 g/L into the culture medium and mix it by vigorous stirring and heating. Then dispense evenly to each tube (~25 mL for tubes of 27 x 195 mm in size).

4. Close tubes with individual plastic caps, or by inserting a cotton wool bung (in this case, cover with a sheet of aluminium foil or moisture-proof paper to prevent the cotton wool from becoming wet) then place in a rack and sterilize at 121°C for 20 minutes.

5. Remove from the autoclave before the agar solidifies and let the agar set.

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

2.3 Bacterial inoculation

1. Prepare inoculum at a concentration of ~5 X 10^8 cfum/mL (OD_{600}=0.5) in sterile water.

2. Select uniform germinated seedlings for inoculation and soak them for 30-60 min into 100 ml rhizobial broth containing around 10^8 cells/ml of appropriate rhizobium strain.

2a. Soak no more than 10 surface-sterilized and germinated seeds with primary roots of 1-2 cm in 10 mL bacterial suspensions in a Petri dish without shaking at room temperature for 0.5-1.0 h. Move the seeds to make sure the roots are completely submerged in the suspensions.

2b. Soak another 10 surface-sterilized and germinated seeds in sterile water for 0.5-1.0 h and use as a negative control.

3. In the laminar flow cabinet, and using sterile tweezers, transfer and plant the pre-germinated seedling adhered by bacteria one into each tube, with the radicle penetrating into the agar (0.6%) in glass tubes (27 x 195 mm) by sterile forceps. Ensure the radicles do not dry out and are oriented downwards. Sometimes it helps to push the tip of the tweezers into the agar to make a crack prior to inserting the radicle. Use the sterile empty glass tubes of the same size to close the tubes containing the seeds in a mouth-to-mouth way. Connect and fix the two tubes by parafilm.

4. Place the tubes in a shallow box or cover the base with aluminium foil to protect the roots from light. Place the tubes in the glasshouse or near a window to allow sufficient light for photosynthesis. Otherwise, place the tubes under the following conditions: 16 h of light 224 (day) and 8 h of dark (night), 4,000 lx, 25 °C and a relative humidity of 54%.

2.4 Nodulation assessment

1. Evaluation of nodulation is performed 20 to 35 days after inoculation.

2. Remove the plants from the rooting medium and note the presence or absence of nodules.

3. Authentication is positive if isolates nodulate the roots of inoculated plants while uninoculated plants remain nodule free. Examine the interior colour of nodules to determine nitrogen fixation efficiency. A strong pink colour indicates the presence of leghemoglobin, necessary for active nitrogen fixation. Brown, white or green nodules are considered ineffective. If the tests are satisfactory, the isolates are regarded as fully authenticated strains.

4. Presence of nodules in the noninoculated treatment invalidates the test and the authentication test must be repeated. Alternatively, crush several nodules from the inoculated and uninoculated controls and reisolate the occupants of nodules and identify by

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

a PCR-based identification technique, such as BOX- or ERIC-PCR that can provide convincing evidence of the identity of the strain in the nodule as described in TRUE-SOP24 and TRUE-SOP30). The DNA fingerprints of the original isolates used as inoculants are compared with the fingerprints of isolates from each nodule to assess their identity.

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973

3 Linked SOPs

SOP Code	SOP Function
EU_TRUE_SOP_024	PCR sequencing analysis to test the presence of the inoculants in the nodules
EU_TRUE_SOP_030	Establishment of protocol for polyphasic characterization

4 Disclaimer

The information presented here has been thoroughly researched and is believed to be accurate and correct. However, the authors cannot be held legally responsible for any errors. There are no warranties, expressed or implied, made with respect to the information provided. The authors will not be liable for any direct, indirect, special, incidental or consequential damages arising out of the use or inability to use the content of this publication.

5 Copyright

6 Citation

Please cite this report as follows:

Efstathiadou E. and Tampakaki A. (2019) Standard Operating Procedure 48: Authentication of rhizobia. Developed by the EU-H2020 project TRUE ('Transition paths to sustainable legume-based systems in Europe'), funded by the European Union's Horizon 2020 Research and Innovation programme under Grant Agreement Number 727973.

This Project has received funding from the European Union´s Horizon 2020 research and innovation programme under grant agreement No 727973