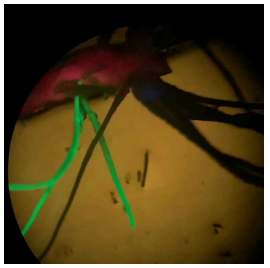
May 21, 2024 Version 3

Hairy root generation in common bean (Phaseolus vulgaris L.) and selection of Agrobacterium rhizogenes clones V.3



PLOS One

Peer-reviewed method

DOI
dx.doi.org/10.17504/protocols.io.261ge3bpjl47/v3

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DOI: https://dx.doi.org/10.17504/protocols.io.261ge3bpjl47/v3

Protocol Citation: Ronal Pacheco, Georgina Estrada-Navarrete, Noreide Nava, Jorge Solis-Miranda, Carmen Quinto 2024. Hairy root generation in common bean (Phaseolus vulgaris L.) and selection of Agrobacterium rhizogenes clones. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.261ge3bpjl47/v3**Version created by **Ronal Pacheco**

Manuscript citation:

Protocol status: Working
We use this protocol and it's working

Created: April 06, 2024

Last Modified: May 21, 2024

Protocol Integer ID: 97873

Keywords: hairy root generation in common bean, generation of transgenic hairy root, hairy roots in common bean, generation of hairy root, hairy root generation, transgenic hairy root, growing hairy root, hairy roots under hydroponic condition, agrobacterium rhizogenes culture, hairy root, selection of agrobacterium rhizogene, agrobacterium rhizogene, mycorrhizal, rhizobial symbiosis, legume, genetic approach, protocol for the generation, common bean, applying genetic approach

Funders Acknowledgements:
UNU-BIOLAC

Disclaimer

Spotlight Video

The video below is a supplement with extra context and tips, as part of the protocols.io Spotlight series, featuring conversations with protocol authors.

https://www.youtube.com/embed/iTnAiDhbtAU?si=w4dcPkyqr8NZPrpl

Abstract

The common bean (*Phaseolus vulgaris* L.) is one of the legumes used to study the molecular mechanisms that regulate mycorrhizal and rhizobial symbioses. To study these mechanisms, the generation of transgenic hairy roots is a very advantageous method for applying genetic approaches. However, the generation of hairy roots is a difficult task that requires a lot of skill and experience. Here we show a new version of an optimized protocol for the generation of hairy roots in common bean. This version includes photos and videos showing the procedure for preparing the *Agrobacterium rhizogenes* culture and the infection procedure, which were not included in the original version. Additionally, we included a step for growing hairy roots under hydroponic conditions.

Protocol materials

- 1.5 mL Eppendorf tubes
- 2 mL Eppendorf
- Plastic Petri dishes (100×15 mm)
- 15 mL Falcon tubes

Troubleshooting

Seeds disinfection (when necessary)

1 Immerse the common bean seeds in [M] 96 % volume ethanol for 00:05:00 and wash them three times with sterile water. 5m

2 Immerse the seeds in [M] 2 % volume sodium hypochlorite for 00:05:00 and wash them three times with sterile water. 5m

Note

The concentration of sodium hypochlorite may be higher than indicated, but this depends on the quality of the seeds. When the quality of the seed is not very good, a higher concentration of sodium hypochlorite can damage a large number of them, rendering them useless for germination.

3 Keep the seeds at 4 °C in a disinfected container, e.g., sterile Plastic Petri dishes (100×15 mm) or reused Petri dishes previously disinfected with [M] 96 % volume ethanol.

Seeds germination (1st day)

4 Placed disinfected seeds, using sterile forceps, in a metal tray on a wet paper towel, previously sterilized in an autoclave. Leave 2 cm between the seeds.

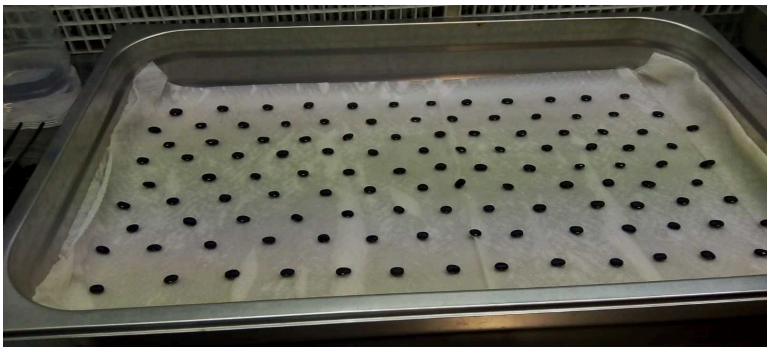


Fig. 1. Orientation of seeds on a wet paper towel, inside the tray.

Note

Paper towels must be moistened with deionized and pre-autoclaved water.

5 Cover the metal tray with aluminum foil and incubate it in a growth chamber at 28 °C for 46:00:00 to 48:00:00 in the dark.

3d 22h

Note

Position the tray at a slight downward angle to improve seed germination. The hilum should face downward. This position causes non-uniform growth of the seeds, to avoid this, the tray should be placed without tilting, which slightly increases germination time.

Fig. 2. Tray containing the seeds, inside the growth chamber.

Preparation of the inoculum of *A. rhizogenes* K599 (2nd day)

6 Spread 150 µL - 200 µL of the inoculum in Petri dishes containing solid LB medium with the appropriate selection antibiotic.

Note

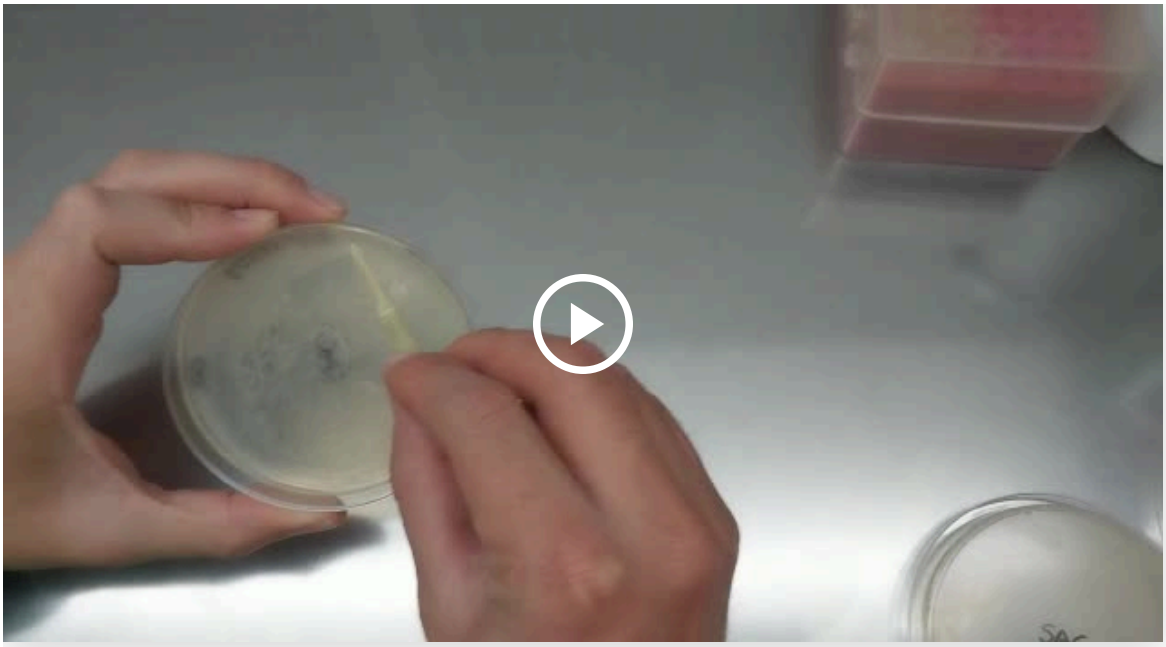
The inoculum consists of a liquid culture of *A. rhizogenes*, transformed with the corresponding vector, and 80% [M] 80 % volume glycerol [M] 50 % (v/v), stored at -80 °C. It is not recommendable to reuse the inoculum.

7 Incubate the Petri dishes inoculated in the previous step, for approximately 30:00:00 at 30 °C. 1d 6h

Note

If after 30 h the *A. rhizogenes* culture has not grown successfully, i.e., the culture layer is dry and very thin, do not use this inoculum for plant transformation and do the following:

7.1 Scratch off this thin layer of dried culture with a sterilized yellow tip or something similar. Transfer this culture to an 1.5 mL Eppendorf tubes or 2 mL Eppendorf



Video 1

7.2 Add LB liquid medium to the Eppendorf tube and centrifugate 8000 rpm, Room temperature, 00:01:00 . Finally, homogenize the content using a micropipette. The content must be viscous, but liquid enough to be pipetted (Fig. 3).

1m

Fig. 3

7.3 Prepare the inoculum in Eppendorf tubes (preferably 0.6 ml) by mixing an equal volume [M] 50 % (v/v) of the liquid content previously obtained, and [M] 80 % volume glycerol. Mix tubes by inversion and immediately place them in liquid nitrogen; finally, store the inoculum at -80 °C.

Note

To use this inoculum, spread 150 µL - 200 µL of the inoculum along with an equal volume of sterile LB liquid medium in Petri dishes containing solid LB medium with the appropriate selection antibiotic. Incubate for approximately 30:00:00 at 30 °C.

Seedling infection by *A. rhizogenes* K599 (3rd day)

8 Expose the infection zone (Video 2, Fig 4).

Video 2.

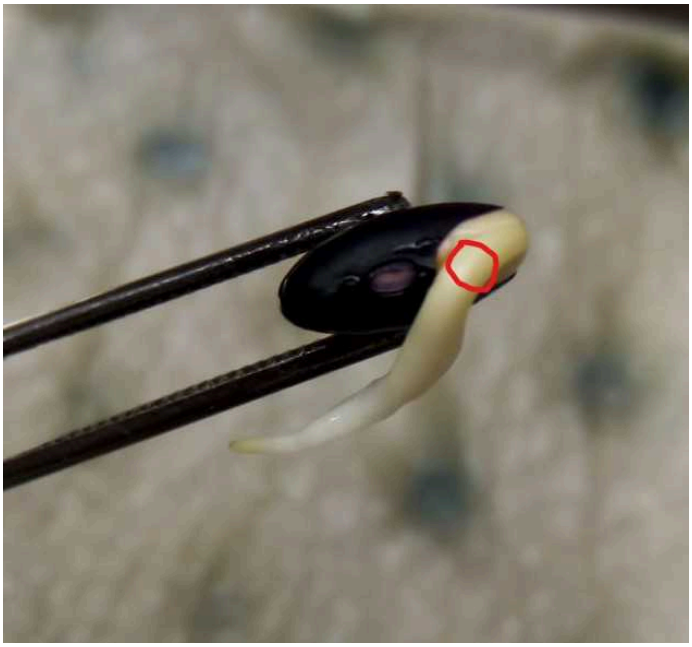


Fig. 4. The red circle indicates the infection zone in the hypocotyl

9 Carefully puncture in the infection zone of the hypocotyl several times using a sterile needle tip (0.4 mm). Apply the inoculum on the wounded zone, directly from the plates, using an autoclaved micropipette tip (Video 3).

Video 3

Generation of hairy roots

10 Place the infected seedlings on the top of plastic tubes, i.e., 15 mL Falcon tubes containing B & D medium (Table 1). Place plastic tubes inside glass tubes containing autoclaved deionized water and cover the glass tubes with plastic caps to prevent water evaporation (Video 3, Fig. 5).

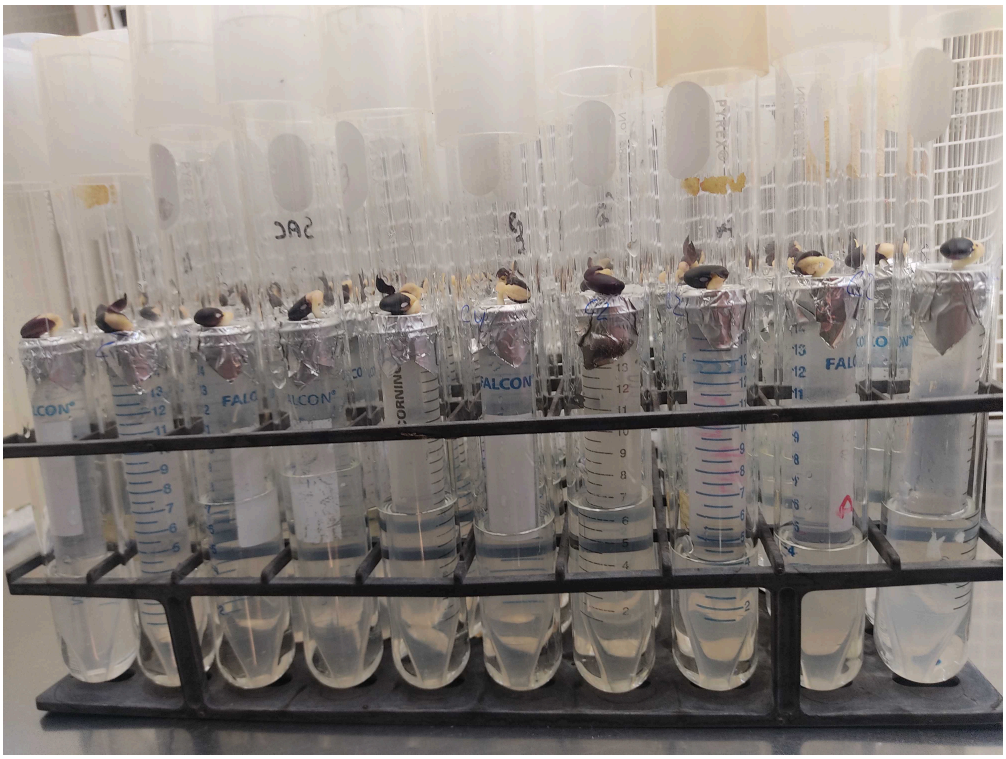


Fig. 5. Plastic tubes, inside glass tubes, containing infected seeds.

Note

A. J. Márquez (Editorial Director). 2005. Lotus japonicus Handbook. pp. 53-82. https://link.springer.com/book/10.1007/1-4020-3735-X

Table 1. Components and preparation of the B & D medium

Stock solutions	Solution component	2000x stock concentration	Concentration in final solution
A	$CaCl_2$	2.0 M	1.0 mM
B	KH_2PO_4	1.0 M	0.5 mM
C	Fe-citrate[b]	0.02 M	10 µM
D	$MgSO_4$	0.5 M	0.25 mM
E	K_2SO_4	0.5 M	0.25 mM
F	$MnSO_4$	2 mM	1.0 µM
G	H_3BO_3	4 mM	2.0 µM
H	$ZnSO_4$	1 mM	0.5 µM
I	$CuSO_4$	0.4 mM	0.2 µM
J	$CoSO_4$	0.2 mM	0.1 µM
K	Na_2MoO_4	0.2 mM	0.1 µM

Table 1. Broughton and Dilworth (1971) nutrient solution. [a]Adjust the pH to 6.8 with KOH. [b]Dissolve Fe-citrate with heating and keep in dark bottle

11 Place the glass tubes on racks and incubate in a growth chamber at around 25 °C, 16:00:00 light/ 08:00:00 8 h dark for 3-5 days post-infection (dpi). 1d

Note

When the first pair of leaves meet the plastic caps (3-5 dpi), remove the caps, and seal the tube hole with parafilm or adhesive plastic (i**mage**). During this period, make sure that the level of water and B & D medium contained within the glass tubes and plastic tubes, respectively, is adequate.
Incubating at a fresh (20-25 °C) temperature is crucial for a high generation efficiency of hairy roots. A higher temperature may reduce the efficiency of hairy root generation.

12 After removing the caps, the plants should be incubated at about 28 °C to promote growthuntil hairy roots emerge (10-13 dpi)

13 Once the hairy roots have emerged, remove the primary root by cutting the stem 2 cm below the hairy root callus.

14 Transfer the seedlings to autoclaved glass tubes containing B & D medium and seal the tube hole with parafilm or adhesive plastic. Maintain plants under this condition for around 72:00:00 at 28 °C to promote hairy root growth.

3d

Note

Make sure the level of the B & D medium is below the hairy root callus, as covering hairy root callus with B & D medium may retard their growth (Fig. 6).


SK

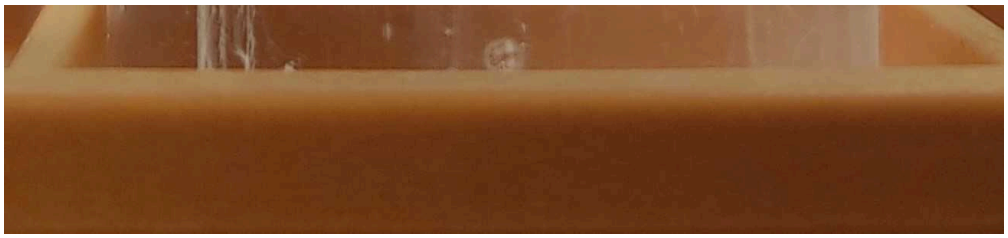


Fig. 6. The red circle indicates hairy root callus

Removal of untransformed roots (selection of suitable plants)

15 Observe the fully developed hairy roots (15 to 16 dpi) using an epifluorescence microscope to remove non-fluorescent roots.

Note

Hairy roots must carry a plasmid containing a fluorescent reporter gene e.g., GFP, RFP, or YFP. Commonly, plasmids for RNAi-based gene silencing or gene overexpression carry a fluorescent reporter gene.

16 Depending on the intended use of the roots, you can proceed as follows.

STEP CASE

Hydroaroponic conditions 2 steps

Hydroaroponic conditions considerably increase hairy root biomass. If a large amount of hairy roots is needed, this method is strongly recommended (Fig. 7). To collect enough root tissue for evaluating the overexpression of silencing capacity of *A. rhizogenes* clones by qPCR, this is the appropiated method.

Fig. 7. Root growth under hydroaroponic conditions

Selection of *A. rhizogenes* clones

17 Extraction of total RNA from hairy roots using an appropriate protocol and cDNA synthesis. For RNA extraction from common bean root tissue, we recommend the following protocol.
dx.doi.org/10.17504/protocols.io.8epv5jq24l1b/v1

Protocol

NAME

RNA extraction from hairy roots of common bean (Phaseolus vulgaris L.) and cDNA synthesis

CREATED BY

Ronal Pacheco

Preview

18 Quantify transcript levels of the gene of interest by qPCR

Note

We recommend the elongator factor 1α (*EF1α*, Phvul.004G075100.1) as a reference gene. If a second reference gene is required, use β-tubulin (Phvul.009G017300.1). For RNAi silencing-based studies, we recommend choosing *A. rhizogenes* clones with a silencing efficiency of at least 70 %.

Protocol references

Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, JE.*et al.* Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*. *Nat Protoc* **2**, 1819–1824 (2007). https://doi.org/10.1038/nprot.2007.259