Agrobacterium tumefaciens-mediated transformation of soybeans using mature cotyledonary node:

Protocol Training

Bayer Russia Biotechnology Conference

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THE TRAINING PURPOSE:

- This presentation is focused on the *A. tumefaciens*-mediated cotyledonary-node transformation method originally reported by Hinchee et al. in 1988.

- All *A. tumefaciens*-mediated transformation methods, the cotyledonary-node method depends on targeting T-DNA delivery into regenerable cells, specifically the meristematic tissue of the cotyledonary node, followed by selection for transgenic cell proliferation and shoot formation.

- Olhoft et al. (2001) demonstrated that inclusion of thiol compounds in the solid cocultivation medium increased T-DNA delivery into the target cells in the cotyledonary node, resulting in increased production of transgenic plants.

- The cotyledonary-node method uses young seedlings for explant preparation, which substantially reduces the costs and labor associated with growing plants for production of immature embryos or maintaining long-term tissue cultures.

- Based on T-DNA delivery, the method has a higher probability of producing simple, non-rearranged transgene loci compared with direct delivery methods.

SAFETY

- Gloves and a lab coat are recommended.
- Disposal of Agrobacterium waste
- All disposable items that have encountered Agrobacterium are considered regulated waste and are disposed of as such in the bins with two orange bags. The orange bags are labeled with permit label containing permit number and transported to a Gaylord for devitalization
- All liquid Agrobacterium waste is devitalized prior to disposal by autoclaving or by adding bleach to make a solution with a final concentration of 10% bleach and allowing the waste to sit for at least 20 minutes. After the bleached material has sat for 20 minutes it may be poured down a laboratory sink with an excess of water.
- Use caution when cutting leaf tissue with razor blades to avoid ergonomic and hand injuries due to sharp blades.
- Discard pipette tips into a beaker lined with a small orange biohazard bag, or clear bag that can be dumped into an orange biohazard bag for bio-waste disposal, in gray tubs.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Media Stock Solutions

All stocks are made with double-distilled water (ddH_2O) and stored at 4°C unless otherwise noted. Filter-sterilize solutions using a 25-mm syringe with a 0.2-µm size pore Acrodisc® filter when noted.

B5 Media

- 10X B5 major salts: 250 mM KNO_3, 10 mM $CaCl_2$, 10 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10mMNaH_2PO_4
- 100XB5 minor salts: 5 mMH_3BO_3, 6 mM$MnSO_4$, 0.7 mM$ZnSO_4$, 0.45 mM KI, 0.1 mM Na_2MoO_4, 0.01 mM$CuSO_4$, 0.01 mM$CoCl_2$
- 100X B5 vitamins: 55 mM myoinositol, 0.8 mM nicotinic acid, 0.5 mM pyridox-ine-HCl, 3 mM thiamine-HCl.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

MS Media

- 10X MS major salts: 20 mM NH_4NO_3, 200 mM KNO_3,30 mM $CaCl_2$,15 mM $MgSO_4$, 12.5mMKH_2PO_4
- 100XMS minor salts: 10 mMH_3BO_3, 10 mM$MnSO_4$, 3 mM$ZnSO_4$, 0.5 mM KI, 0.1 mM Na_2MoO_4, 0.01 mM$CuSO_4$, 0.01 mM$CoCl_2$
- 100X MSIII iron: 10 mM $FeSO_4$, 10 mM NaEDTA. Dissolve $FeSO_4$ in 200 mL ddH_2O and in a separate container, boil EDTA for 2 min in 200 mL ddH_2O. Slowly combine the two solutions and cool to room temperature before bringing the final volume to 1 L

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Plant Growth Regulators

- Indole-3-acetic acid (IAA): 1 mg/mL stock solution. Dissolve 20 mg in 20 mL 95% ethyl alcohol. Make 1-mL aliquots and store at -20°C

- Gibberellic acid (GA_3): 1 mg/mL stock solution. Dissolve 50 mg GA_3 in 50 mL 70% ethyl alcohol. Store at 4°C

- 6-Benzyl-aminopurine (BAP): 1 mg/mL stock solution. Dissolve 100 mg BAP in *0.2 N*HCl; bring solution up to 100 mL with ddH_2O. Filter-sterilize. Store at 4°C

- trans-Zeatin riboside (ZR): 1 mg/mL stock solution. Dissolve 50 mg ZR in 0.2 N HCl; bring solution up to 50 mL with ddH_2O. Filter-sterilize. Store at -20°C

- Indole-3-butyric acid (IBA): 1 mg/mL stock solution. Dissolve 20 mg IBA in 20 mL 95% ethyl alcohol. Store at -20°C

- Paromomycin. 100 mg /ml. Dissolve in water/KOH. Filter-sterilize. Store at -20°C.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

A. tumefaciens Strains

C58, AGL1, EHA101, EHA105, GV3101, and LBA4404 transformed with a range of binary plasmids have been used for soybean transformation using the cotyledonary node method. The method described here has been optimized for NPTII selection.

Plant Materials

Field-grown, disease-free soybean seeds are used for explant preparation. **We use the cultivar Williams 82.**

1 day after germination

5 days after germination

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Soybean Tissue Culture Media

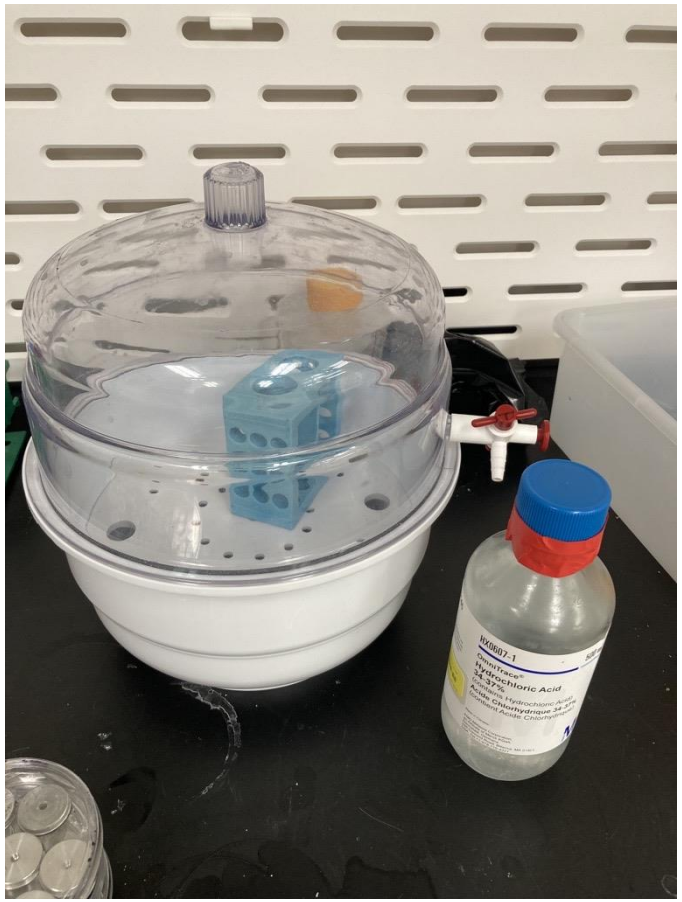
- Germination medium (GM): full-strength B5 major and minor salts, MSIII iron, B5 vitamins, 2% sucrose, 4g/L activated charcoal, adjusted to pH 5.8 with 1N KOH, solidified with 0.8% Noble Agar (Difco), autoclaved, and poured into 25×100-mm Petri dishes.

PROCEDURE

Seed Sterilization and Seedling Growth

1. Chorine gas seed sterilization:

a. Place two layers of seeds in a 15 x 100-mm plastic Petri dish.
b. In an exhaust fume hood, place seed into a glass desiccator with a 250-mL beaker containing 100 mL bleach (5% NaOCl) and slowly add 3.5 mL 12 *N* HCl to the beaker.
c. Seal the lid on the desiccator and sterilize the seeds for at least 24 h. For extremely contaminated seeds, repeat this procedure.
d. Seeds can be stored in sealed Petri plates until use.



2. Bleach seed sterilization

a. Sterilize in 50% commercial bleach plus few drops of Tween 20 - 20-30 min.
b. Wash 3-4 times with sterile water.

Seed Sterilization and Seedling Growth

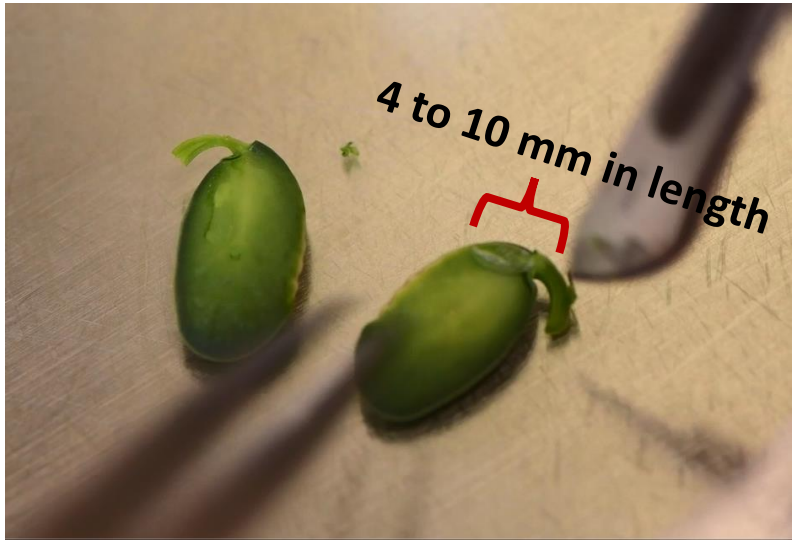
1 day after germination

5 days after germination

Germination in vented plastic bag to maintain humidity

Seed Sterilization and Seedling Growth

Germinate approx. 16 seedlings per Petri plate containing GM at 25°C under 18-h light/6-h dark cycle at 90 to 150 µE/m^2/s and grow until the cotyledons turn green but before the first true leaves grow completely out of the cotyledon *(see* **Note 2).** The seedlings can be used immediately for transformation or stored at 4°C overnight or until the *A. tumefaciens* is ready for inoculation. We have found that seedlings are susceptible for transformation even after 5 d at 4°C.



Note 2: The actual length of time on GM is dependent on numerous factors including genotype, health of the seed, and light intensity. Normally it takes anywhere from 3 d for small-seeded cultivars to 5 to 7 d for large-seeded cultivars. A good indicator of optimal explant development, is when **the first true leaves are 4 to 10 mm in length**.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Culture Media *for A. tumefaciens*

- Yeast extract peptone (YEP) Medium: 1% Bacto-peptone (Difco), 0.5% yeast-extract (Difco), 0.5% NaCl, adjusted to pH 7.0 with 1 N NaOH, solidified with

- 1.2% granulated agar (Difco) and poured into 15×100-mm Petri dishes. Liquid cocultivation medium (LCCM): 1/10 strength B5 major and minor salts, 1/10 MSIII iron, 3% sucrose, and 3.9 g/L 2-[N-morpholino]ethanesulfonic acid (MES), adjusted to pH 5.4 with 10 N KOH. In a separate beaker, dissolve 40 mg acetosyringone in 5 mL 95% ethyl alcohol and add 10 mL 100X B5 vitamin stock, 0.25 *µL* GA_3 stock, and 1.67 mL BAP stock per liter of basic medium and filter-sterilize. Add the filter-sterilized solution to cooled LCCM.

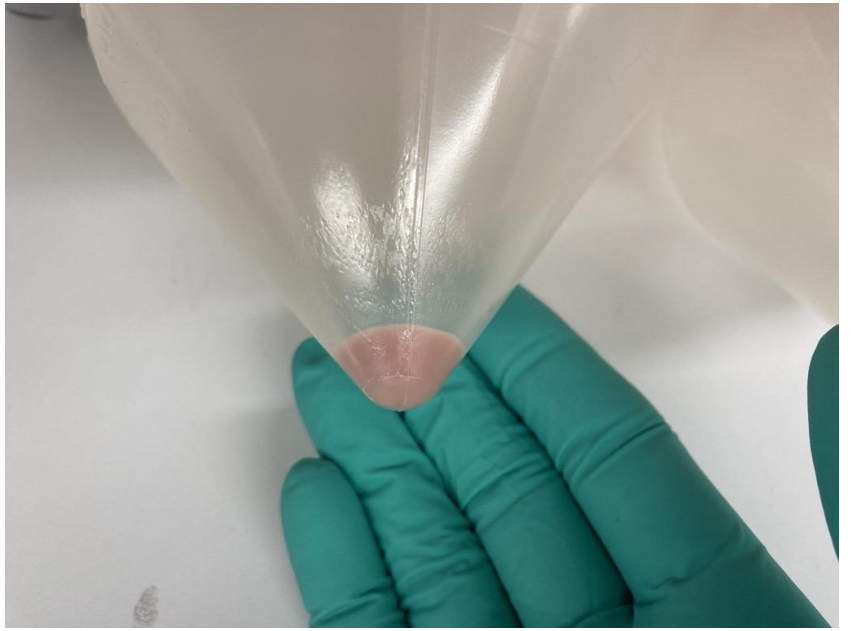
A. tumefaciens Preparation

1. One day before explant inoculation, add 250uL of *A. tumefaciens* glycerol stocks plus appropriate antibiotics to 200 mL YEP liquid medium in a 500-mL Erlenmeyer flask. Incubate the flask overnight at 25°C (125 rpm) until an OD_{600} between 0.8 and 1.0 is reached.

2. Before preparing and inoculating soybean explants, divide the broth into 50-mL or 250-mL aliquots and pellet A. *tumefaciens* by centrifugation for 10 min at 3270g at 20°C. Resuspend the pellet in 25 mL liquid CCM and place at room temperature at least 30 min before use. This makes the final OD_{600} approximately 1.0.

Glycerol stock

Pelleted Agro – note peach color



REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Solid cocultivation medium (SCCM):

- Autoclave 1/10 strength B5 major and minor salts, 1/10 strength MSIII iron, B5 vitamins, 3.9 g/L MES, 3% sucrose, adjusted to pH 5.4 with 10 N KOH and solidified with 0.5% Noble Agar.

- In a separate beaker, dissolve 40 mg acetosyringone in 5 mL 95% ethyl alcohol and add 10 mL 100X B5 vitamin stock, 0.25 µL GA_3 stock, and 1.67 mL BAP stock per liter of basic medium and filter-sterilize.
- Prepare three separate solutions of 1000 mg/L L-cysteine (final concentration 8.8 mM), 158 mg/L sodium thiolsulfate (1 mM), and 154 mg/L dithiothreitol (1 mM). Each thiol compound is dissolved in 5 mL water per liter and kept separate from one another during preparation to reduce the sulfur odor (*see* **Note 1**).
- Add acetosyringone solution and thiol compound solutions to cooled SCCM and pour into 15×100-mm Petri dishes.

- After medium solidifies, place a single sterile Whatman #1 (70-mm) filter paper (Whatman International, Maidstone, England) on the medium in each plate.

Explant Preparation and Inoculation

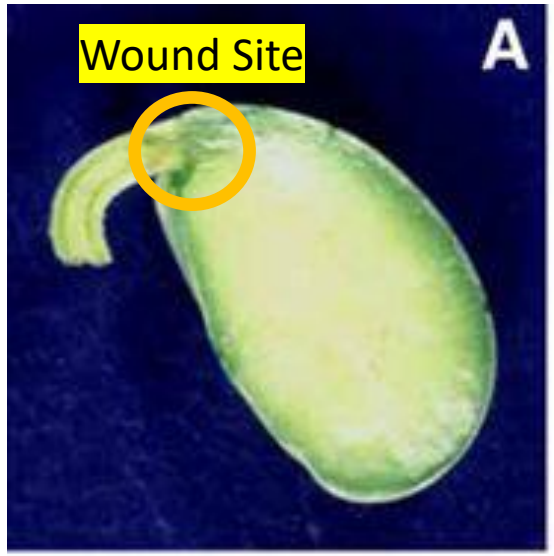
1. After the *A. tumefaciens* is resuspended in 25 mL liquid CCM, begin preparing the soybean cotyledonary nodes.

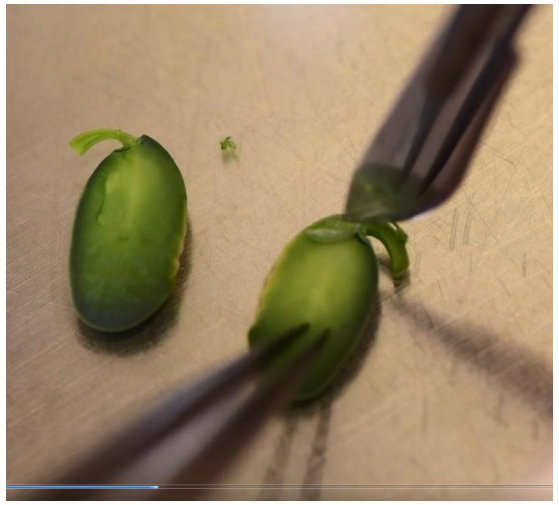
2. Excise the root and the majority of the hypocotyl approximately 3 to 5 mm below the cotyledonary node using a number **15 Personna Plus surgeon's blade**. Bisect the cotyledons by cutting vertically through the hypocotyl region to produce two equal halves, each containing a cotyledon, half of the hypocotyl, and half of the epicotyl tissue.

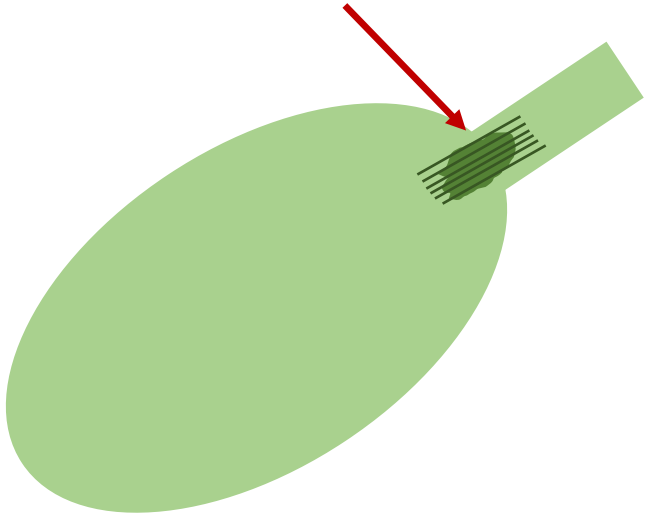
3. Remove the epicotyl at the base of the cotyledonary node by a single cut. In addition, remove all preformed axillary shoots (easily identified as pubescent vegetative growth), taking care to avoid damaging meristem cells located within this tissue. Removing the epicotyl and preformed axillary shoots suppresses apical dominance, thereby inducing *de novo* proliferation from the axillary meristems.

Explant Preparation and Inoculation

4. Finally, wound the axillary meristems and the cotyledonary node by slicing approx. 10 times with the blade perpendicular to the hypocotyl. When wounding, cut deep enough to access the meristematic tissue but avoid removing or extensively damaging the tissue (*see* **Note 5**). **Figure 1A** shows an example of an explant ready for *A. tumefaciens* inoculation.







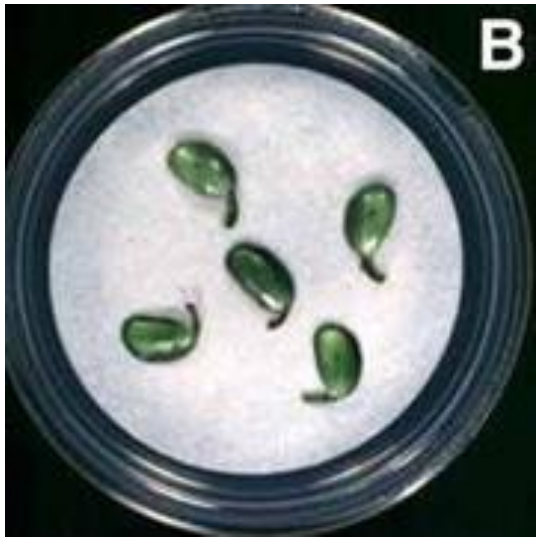
Note 5: It is best to dissect the soybean explants under magnification. The target cells are located in the tissue between the hypocotyl and the cotyledon, which can be hard to reach with the scalpel. However, on a good explant, one can see circular tissue growth around the target tissue.

Explant Preparation and Inoculation

5. About 50 explants are transferred to a Petri dish containing 25 mL of CCM/A. *tumefaciens* suspension for approximately 30 min. Explants can be moved to the suspension either after each wounding or after a group of explants are wounded. (We cut approx 50 before moving.)

6. After inoculation, five explants are plated adaxial or wounded side down on the Whatman paper overlaying SCCM (**Figure 1B**). Filter papers prevent A. *tumefaciens* overgrowth on the soybean explants. Wrap five plates with Parafilm “M” (American National Can, Chicago, IL) and incubate for 5 d in the dark at 25°C (*see* **Note 6**).

Wound site

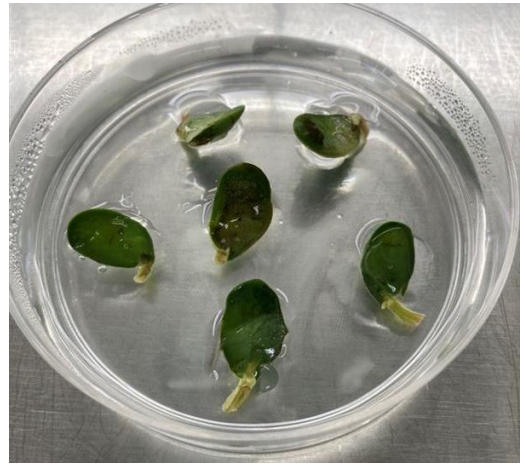


Note 6: We find that infection is greater after 4 or 5 d of incubation rather than 1 to 3 d. *A. tumefaciens* overgrowth is not a problem with long incubations using this explant tissue.

Selection and Plant Regeneration

1. Excess *A. tumefaciens* is removed by briefly immersing the explants into liquid SIM (*see* **Note 7**).

2. Rinsed explants (five per plate) are perpendicularly imbedded hypocotyl end down into solid SIM not containing hygromycin B so that the hypocotyl and wounded cotyledonary-node tissue are under the medium surface (*see* **Note 8**). Wrap plates with Scotch 394 venting tape (3M, St. Paul, MN) and place in a growth chamber for 14 d at 25°C under an 18-h light/6-h dark cycle at 90 to 150 uE/m_2/s (*see* **Note 9**).



Note 7: At this time in the protocol, it is helpful to sample the explants for transient GUS expression if the *gusA* gene was on the binary vector used for transformation. Although not necessarily an accurate indicator of transformation efficiency, this test helps to determine whether there were any overall problems with transformation. In a good transformation experiment, 80 to 100% of explants will have transient GUS expression on the hypocotyls, cotyledonary-node region, and/or cotyledons.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

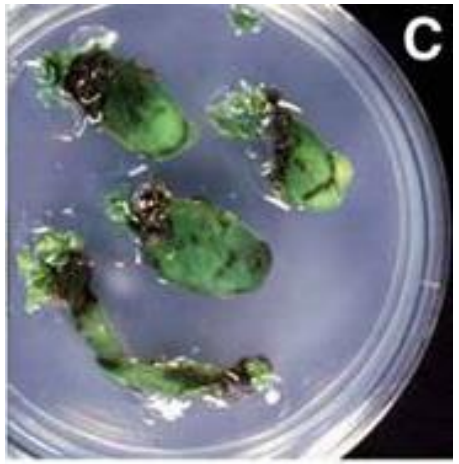
Shoot induction medium (SIM):

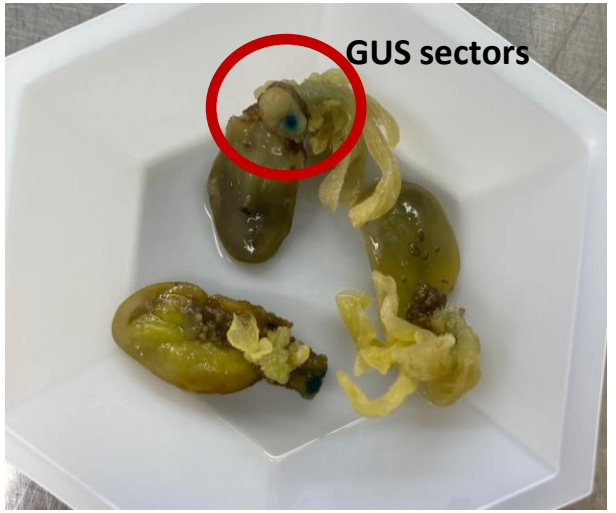
- Autoclave full-strength B5 major and minor salts, MSIII iron, 3% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 10 N KOH and solidified with 0.8% Noble Agar (Difco).
- Mix together 10 mL 100X B5 vitamins, 1.67 mL BAP, 500 mg ticarcillin (TICAR), 100 mg cefotaxime (Claforan).
- Filter-sterilize mixture with a 25-mm syringe filter with a 0.2-$_{\mu}$m size pore Acrodisc filter.
- Add Paromomycin when appropriate.
- Add the sterilized mixture to the cooled medium and pour into 25×100-mm Petri dishes.

Selection and Plant Regeneration

3. Transfer explants to SIM containing 50 mg/L paromomycin after carefully excising the hypocotyl. Also remove very long shoots since they arose from preformed shoots that were not removed during explant preparation. Incubate in the growth chamber for an additional 14 d.

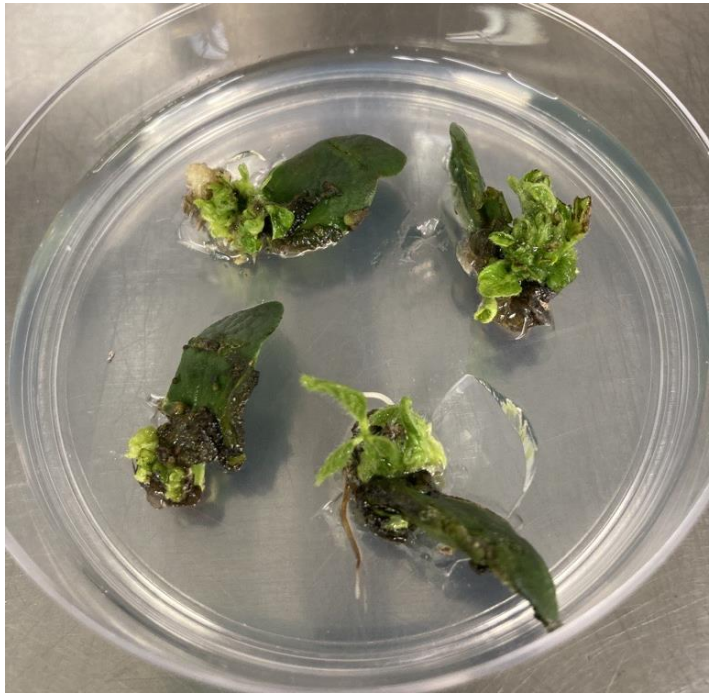
4. After 28 d on SIM (Fig.1C), remove the cotyledon and any dead tissue and transfer explants to SEM containing 50 mg/L paromomycin. Incubate in growth chamber for an additional 14 d (*see* Note 10 and Note 11).





4 weeks on shoot induction media
2 weeks no selection;
2 weeks 200mg/L paromomycin

Selection and Plant Regeneration – additional pictures



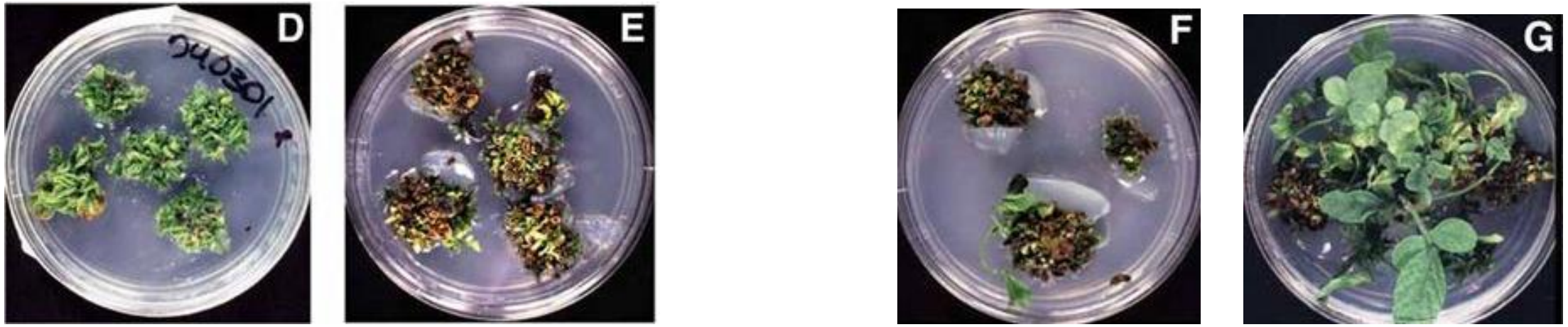
REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Shoot elongation medium (SEM):

- Autoclave full-strength MS major and minor salts, MSIII iron, 3% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 10 N KOH and solidified with 0.8% Noble Agar (Difco).
- Mix together 10 mL 100X B5 vitamins, 50 mg L-asparagine, 100 mg L-pyroglutamic acid, 0.1 mL IAA, 0.5 mL GA_3, 1 mL ZR, 500 mg ticarcillin, and 100 mg cefotaxime.
- Filter-sterilize mixture using a 25-mm syringe filter with a 0.2-μm filter.
- Add the mixture to the cooled medium and pour into 25×100-mm Petri dishes.

Selection and Plant Regeneration

5. After every 2 to 3 wk, transfer explants to fresh **SEM** medium after excising dead tissue. The explants should hold together and not fragment into pieces and should retain somewhat healthy sectors up until 8 wk after cocultivation (4 wk on SEM; **Fig.1D** and **Fig.1E**). After this time, it is normal that many shoots are lost and some explants turn a caramel brown color; however, there should also be some explants with healthy vigorous shoot elongation at this time, as seen in **Fig.1F** and **Fig.1G** (*see* **Note 12**).



Note 12: Using thiol compounds in the cocultivation medium leads to an increase in transgenic cells in the explants, especially on any surface that was initially cut before inoculation. When you are using other methods for selection, such as PPT or glufosinate selection, it may be necessary to increase the concentration of selective agent used over explants not treated with thiol compounds during cocultivation to kill the nontransgenic cells in the callus/shoot mass effectively.

REQUIRED OR REFERENCED MATERIALS, EQUIPMENT, AND DOCUMENTS

Rooting medium (RM):

- Autoclave $^{1}/_{2}$-strength B5 major and minor salts, full-strength MSIII iron, 2% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 1 N KOH and solidified with 0.8% Noble Agar (Difco).

- Add 1 mL IBA stock to the cooled medium and pour into sterile vials.

Other Supplies and Materials

- Number 15 Personna Plus surgeon’s blade (American Safety Razor Company, Staunton, VA).

- For greenhouse supplies, bags of perlite and peat moss from any manufacturer, pasteurized soil (sandy loam preferred), and 2.5-gallon pots or larger. A slow-release fertilizer (15-9-12) or liquid fertilizer (21-5-12) can be used when needed.

Selection and Plant Regeneration

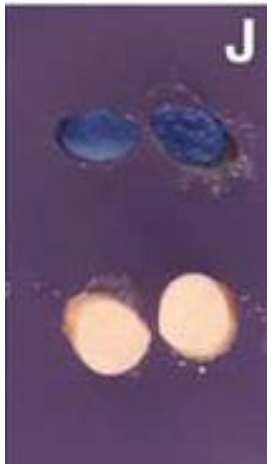
6. To increase shoot production from a promising explant, push the developing shoot cluster into the medium to induce further axillary shoot growth from the nodes. In several weeks, most of these shoot clusters will develop into proliferating shoot cultures that are clones of one another.

7. Healthy, vigorous shoots with at least three sets of leaves that are preferably over 4 cm in length are excised and plated on RM (**Fig.1H**). In a typical experiment at least 90% of the shoots form roots at the cut site after 5 to 14 d on RM. It is common that roots also form while the shoots are still in SEM. When this happens, transfer to RM for several days before transferring to the greenhouse.

Transplantation and Greenhouse Maturation

1. Once a small root is formed on the shoot, the plantlets are transferred directly to large pots (2.5 gallons or larger) filled with a mix of 1/2 pasteurized top soil (sandy loam): 1/4 perlite: 1/4 peat moss in the greenhouse. We have found that a harden-ing-off period in a smaller pot is not necessary in our hands, and it is not worth the time it takes to do two transfers. The greenhouse conditions are set to 28°C with a 16/8-h (light/dark) photoperiod (see Note 9) under natural lights supplemented with 1000-W high-pressure sodium lamps. We try to provide a minimum of 200 µmol/m2/s of light.

2. To reduce moisture loss and to protect the shoot from the surrounding environment, cover the plantlets with a clear plastic container and remove when the first set of new leaves opens. When environmental conditions are optimal, greater than 90% of the rooted shoots develop into healthy, fertile plants.



The seed-set for T_0 soybean plants is highly variable and, when plants are grown in a greenhouse, is sensitive to the time of year (photoperiod and light intensity), cultivar, maturity zone of greenhouse, and environmental pressures (especially pests). Plants may produce as little as one seed to hundreds of seeds, so one should take into consideration what photoperiod is optimal for each environment and use supplemental lighting or shading when needed. Using this method, the typical maturation period is approximately 3 to 4 mo.

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