Germination, growth and nodulation of *Medicago truncatula*

This website provides information about protocols used in the Long Lab. For additional information and protocols, refer to the *Medicago truncatula* Handbook at http://www.noble.org/medicagohandbook/

Media preparation

In our lab, we use modified buffered nod medium (BNM—see recipe below) at pH 6.0 for alfalfa and pH 6.5 for *Medicago truncatula*.

M. truncatula is sensitive to ethylene. It grows poorly, exhibits the thick, short-root phenotype, excessively hairy roots, and it nodulates poorly. The addition of the ethylene synthesis inhibitor AVG (2-aminoethoxyvinyl glycine, Sigma) at 0.1 - 0.5 μM, and the use of purified agar (Sigma) have been useful in promoting healthier root growth. We make a 2 mM AVG stock, filter sterilize, and freeze in aliquots.

BNM + AVG	Amount per L
$CaSO_4 \cdot 2H_2O$	344 mg
MES	390 mg
Nod Majors (200x stock)	5 ml
Nod Minors I (200x stock)	5 ml
Nod Minors II (200x stock)	5 ml
Fe-EDTA (200x stock)	5 ml

1. Add ingredients to ~750 mls distilled water and stir to dissolve
2. pH to 6.5 with KOH
3. Add dH_20 to total volume of 1 L
4. Add Sigma Purified Agar 11.5 g
5. Autoclave, cool in 55°C water bath
6. Add AVG to 0.1-0.5 uM (I've had good success with 0.1)
7. Pour 250 mls per large plate (more plates/L may be poured for very short term plant growth)

Stock solutions for BNM

200x Fe-EDTA	Amount per L
Na_2EDTA	3.73 g
$FeSO4 \cdot 7H_20$	2.78 g

200x Nod Majors	
$MgSO_4 \cdot 7H_2O$	24.4 g
KH_2PO_4	13.6 g

200x Nod Minors	Amount per L
$ZnSO_4 \cdot 7H_2O$	920 mg
H_3BO_3	620 mg
$MnSO_4 \cdot H_2O$	1.69 g

200x Nod Minors II	
$Na_2MoO4 \cdot 2H_2O$	50.0 mg
$CuSO_4$	3.2 mg
$CoCl_2 \cdot 6H_2O$	5.0 mg

Containers

For in vitro experiments we use slant tubes (#47729-572, VWR) capped with translucsent Kim Kaps (#731480, Carolina Biological), or 24x24 cm assay plates (Applied Scientific #AS-72075).

Plants may also be grown in Culture tubes or Cone-tainer® tubes (Stuewe & Sons) containing vermiculite, potting mix, or Turface® (calcined clay particles). Cone-tainers are open to the air and are not sterile.

Preparing slant tubes

1. Add filter-sterilized AVG to the autoclaved, cooled medium in a flask
2. Deliver 8 mls medium to each tube via an automatic dispenser with autoclavable parts
3. Tilt rack to form slants (agar should come to about 1 inch below tube rim)

1. Alternatively, deliver 8 mls media without AVG into each tube
2. Cap loosely, autoclave and cool slightly but do not allow to solidify
3. Add AVG to each tube using a repeating pipetter
4. Shake tubes (carefully) to mix, and tilt racks to form the slants

NOTES: Cap tubes loosely after plants have been added to allow for maximum air exchange
Shade plant roots with foil wrapped around the rack
Alfalfa does not require AVG
Our dispenser is an Oxford 470—very old—so you may have to find a modern alternative.
I found one at Tritech Research http://www.tritechresearch.com/pourboy.html

Preparing Square Assay Plates
Add AVG to autoclaved medium that has been cooled in a 55°C water bath
Pour 4 plates per liter (up to 8 plates per liter if the plants are to be kept less than 2 weeks)

NOTE: Assay plates are expensive, so we often reuse ours after cleaning and sterilizing in Clorox.

Preparing vermiculite tubes
Add vermiculite to within two inches below tube rim
Cap loosely and autoclave
Add 8 mls sterilized liquid BNM + AVG to each tube

Growth Chamber Conditions

Light: 200-600 µmol/m^2/s.
Temperature: 20°C +/- 2 °C
Day/night cycle: 16/8

Seed Sterilization

Medicago truncatula seeds must be scarified (the seed coat must be breached) for imbibation and germination to take place. The *Medicago truncatula* Handbook has additional information on the subject of dormancy at http://www.noble.org/medicagohandbook/

Sulfuric Acid Scarification

1. Scarify seeds in small flasks with enough concentrated sulfuric acid to cover for 5-12 minutes (we use the shorter time for sensitive mutants)
2. Decant acid into a hazardous waste bottle
3. Rinse seeds with sterile water 4-5 times, putting first rinse in the hazardous waste bottle
4. Surface sterilize in concentrated Clorox for 2 minutes
5. Add at least an equal volume of water and sterilize for 1 minute more
    a. If you have several small flasks of seeds, you may start decanting or pipetting off the liquid at this point (it will take time to get through the flasks)
6. Decant liquid into sink with running water
7. Rinse with sterile water 4-5 times
8. Imbibe at room temp on an orbital shaker for 3-6 hours, changing sterile water several times
9. At this point seeds can plated for germination or can be stored in water at 4^{o}C for 24-72 hours to improve germination, especially for recently harvested seed).

NOTES: Several rinses are needed to remove the Clorox, but it is possible to use a shorter time or even skip imbibing on the shaker, especially if seeds are to be put in the cold.

For older seeds

If seeds are >1 yr old, they do not need to be scarified and the sterilization steps are modified as follows:

1. Swirl seeds on shaker (seeds move slightly) with enough 70% EtOH to cover for 30 minutes
2. Rinse 4-5 times with sterile distilled water
3. Add full-strength Clorox and swirl gently on orbital shaker for 30 minutes
4. Add at least an equal volume of sterile water, mix and decant (many seeds will float—you may have to pipet liquid off)
5. Rinse with sterile water 4 times and continue with imbibing procedure (step 8 above)

NOTE: This procedure is hard on seeds and is recommended only if you have a large stock of seed. We lose many seeds; some due to lost viability of old seed and some due to the harshness of the procedure.

For seeds not needing steriliaztion

If sterile plants are not needed, seeds may be scarified by rubbing them lightly between two pieces of sandpaper. Germinate in Petri plates containing a small amount of water or plant directly in soil after scarification (germination may not be as synchronous with direct planting).

Seed Germination

1. Pour sterile seeds and water into a sterile, plastic Petri plate, and swirl to distribute seeds
2. Decant most of the water, then remove the rest with a pipetman or Pasteur pipet
3. Invert plate and seal with Parafilm
    a. Seeds will stick to bottom of plate (now the "top")
4. Germinate overnight in the dark at room temperature
    a. Germination in a 28-30^{o}C warm room may improve germination
5. Plate sprouts the next day
    a. Roots should be 1-2 cm long after 18 hours

NOTES: Precious seeds that have not germinated may be left in the plates and plated as they geminate for 2-3 consequtive days. Rinse with sterile water, and follow steps 3 and 4 again.

Plating germinated sprouts

Work in a laminar flow hood if one is available. Sterilize utensils by dipping them in 70% ethanol and passing them through a flame to ignite the alcohol. Resterilize frequently during work. Be sure utensils are not too hot when you touch the plant tissue with them. *Be sure to keep the root tips moist—they will dry out in the air stream from the flow hood, so replace the plate lid in between transfers if you are slow.*

1. Add a small amount of sterile dH_20 to plate and swirl to loosen sprouts and immerse roots
2. Using blunt, nonserrated-tip forceps, gently pick up a sprout just under the cotyledons
3. Transfer sprouts to BNM plates or tubes
    a. On plates, lay 10-30 sprouts in a row 5-10 cm from the plate top, root tips down
    b. For tubes, use mini-spatula to transfer sprouts to agar, 1 inch down from tube rim
4. Seal plate bottom with Parafilm and then all four sides with 3M Micropore Surgical Tape (Professional Hospital Supplies cat. #102-910)
5. Set plate upright so root tips are pointing down while working on next plate
    a. Roots grow quickly and will turn down into the agar if plates are left lying flat
6. When all plates are finished, place them upright in a large plastic bin with sides at least 4-6” high
    a. Plates may be angled back *slightly*, but if angle is too extreme, roots will grow into agar
7. Shade roots on front and back (and in between plates, if desired)
    a. Some people wrap the lower ¾ of their plates in foil
    b. Others use the white papers that come with the packages for shading
    c. I use exposed x-ray film between plates, and cover the front and back with white paper to keep down heat from the lights

Inoculation of *Medicago truncatula* Seedlings with *Rhizobium meliloti*

We inoculate roots with Rhizobia 2 to 5 days post plating. Two inoculation procedures are used in the lab—spot inoculation, where 1 µl of culture is delivered onto each root tip, and flood inoculation, where 5-10 mls of culture is washed over the roots and the excess is removed.

Flood inoculation

1. Prepare an overnight culture of *Rhizobium meliloti* in LB or TY + appropriate antibiotic
    a. 24-27 hours with vigorous shaking at 28-30^{o}C
    b. OD_{600} of RM1021 will be 1.5 – 2.5, depending on how heavily you inoculate
2. Pellet bacteria and wash x2 in ½ x liquid BNM (no additives or agar) or in 10mM sterile $MgSO_4$
3. Spec at OD_{600} and dilute back to 0.05
4. Using a pipette, dribble 5-10 mls of inoculum across the root nodulation zone (10-30 plants)
5. Jiggle the plate so liquid covers the roots well
6. Wait 5 minutes with plates lying flat, then tilt plate up so excess liquid runs to bottom
7. Pipette off excess liquid that collects in the bottom of the plate
8. Replace lid and set plate upright after inoculation until all plates are done
    a. This is important—it helps keep roots from growing into the agar
9. Reseal all plates and return to growth chamber

Spot Inoculation

1. Follow steps 1-3 as for flood inoculuation
    a. A microfuge tube is a convenient size for the small amount of inoculum needed
2. Using a 2-µl pipetman, deliver 1 µl inoculum to each root tip
    a. Push out the inoculum so a drop forms at the pipet tip, then touch it to the root tip
    b. The liquid will travel up the root to the competent zone
3. Replace lid and set plate upright after inoculation until all plates are done
4. Reseal all plates and return to growth chamber

NOTES: It is useful to mark root tips with a dot on the back of the plate (or side of the tube) at inoculation time as a reference for later data collection. A felt-tipped pen works fine for this.

A broad range of bacterial concentrations will work for either method. You may simply dilute your bacteria 1:50 if you want to see nodules but are not concerned with replicating experimental conditions.

What to expect

On *M. truncatula* with good conditions you can start seeing nodule primordia 4-5 days after inoculation with *R. meliloti*. Mature nodules may be scored 14 – 28 days post inoculation (dpi). There will be more immature nodules at the earlier time point. Plates may be kept for up to six weeks, but plants do not thrive after the first few weeks and should be taken by 4 weeks at the latest if they are to be transplanted.

Moving plants from plates to pots

Plants should be "hardened off" by slow exposure to their new environment. The following is a method that has worked well in our lab. It may be sped up as long as humidity is lowered in increments. I have also planted directly in a soil-filled pot and covered the plant with a magenta box to maintain humidity.

1. Fill Magenta Box fitted with plastic connectors 1/2 full with moist soil mix. Attach another magenta box upside-down to the connector and autoclave.
2. Cool, and carefully transplant selected plant from the plate to the box.
3. Replace lid and move box to growth chamber or greenhouse.
4. After a week, water lightly with 1/4 strength fertilizer such as Miracle Gro, Peters or Plantex, and lift top Magenta Box so it is loose, but leave it sitting on lower box to cover the opening.
5. After 2 days, move the top boxes slightly to create an air link to the outside.
6. Continue to increase the size of the open space over a period of about a week.
7. Transfer to pot when plant has become acclimated to the new environment.