GREENHOUSE SCREENING PROTOCOL OF COMMON BEAN FOR NODULATION AND NITROGEN FIXATION

Materials needed:
2:1, v/v Perlite:vermiculite potting media
Plastic containers
Rhizobia inoculant, liquid or peat based
2% bleach solution
Sterile water
Nitrogen free nutrient solution (Broughton and Dilworth, 1970)

1) Sterilize perlite:vermiculite mix. Fill clean plastic pots with perlite:vermiculite mix.
2) Sterilize bean seeds by soaking for 2 min in 2% bleach solution, rinse twice, 2 min each with sterile water.
3) If using peat based inoculant, coat moist seeds with inoculant by swirling them in a slurry of the inoculant and sterile water. If using a liquid broth culture, place seeds in the liquid culture and agitate to ensure thorough coverage.
4) Plant seeds in perlite:vermiculite potting mix, placing seeds 1 cm below surface.
5) Water seeds to settle potting mix.
6) As needed, apply nitrogen free solution to containers to maintain proper root moisture. Approximately 200 ml is enough to wet the media, with a small amount draining through the container. The pH of leachate should be monitored to maintain a pH of between 6.0 and 6.5 to not adversely affect nodulation. Phosphoric acid may be used to adjust the pH of the nutrient solution.

At first flower, plants are harvested. Harvest will occur over a period of several days as each genotype reaches flowering stage.

1) After measuring the height of the plant, remove top portion of plant by severing with a blade or scissors at the potting medium line. Dry plant tops at 60 C to measure biomass and nitrogen content.
2) Invert pot and allow root ball to fall free from the pot. Gently shake the root ball with your hands to remove as much of the medium possible while not breaking any roots or dislodging nodules.
3) In a bucket of clean water, submerge the root system and agitate to remove remaining perlite and vermiculite. Gently moving the root mass in the water may help to remove more perlite and vermiculite.
4) Remove roots from water and gently shake roots to dislodge any remaining perlite, perlite may gently be picked off without causing damage to the roots.

5) Roots are laid out and spread to their maximum length; they may have coiled in the pot. Measure roots from base of stem to tip of the longest root. Photograph roots while spread for future analysis.
6) Nodules may be evaluated at this time. To assess whether nodules are fixing nitrogen, break one open to see what color it is inside. Nodules which are orange or red are fixing nitrogen; those that are green, gray, or white are not fixing nitrogen. Fixing nodules often appear orange without the need to break them open.

Protocol for culturing *Rhizobium* spp.

Materials:

Yeast Mannitol Agar

Petri Dishes

Inoculating loop

Incubator, set to 28 C

1) Mix yeast mannitol agar (YMA), recipe below, by heating to dissolve agar. One L of YMA should be split into two-1 L Erlenmeyer flasks. Flasks should be stopped with cotton wrapped in cheese cloth. A square of foil is then wrapped over the top. Autoclave for 25 minutes at 25psi.
2) Allow YMA to cool enough to handle, but not solidify. Under a sterile hood, fill bottom of petri dish enough to cover the entire surface, replace the top of the petri dish. Allow petri dishes to cool and solidify in the hood. If not used immediately, petri dishes may be wrapped in the plastic sleeve the petri dishes were packaged in and refrigerated until needed. Note: cloudiness during storage indicates contamination and plates should not be used.
3) Over a Bunsen burner heat an inoculating loop until it glows orange. Cool by placing loop in the YMA of the dish you are using.
4) Insert loop into source of rhizobia and apply to surface of the YMA in a serpentine pattern to spread the rhizobia over the plate. Sources of rhizobia could be: another colony on YMA, a colony isolated from a nodule or soil and growing in isolation media, a vial containing glycerol with a sample, lyophilized culture which has been rehydrated, or a liquid culture.
5) Place culture in an incubator. Colonies should be subcultured to maintain active growth while using for screening every one to three days.

Nitrogen Free Nutrient Solution, from Broughton and Dilworth, 1970

Stock Solution 1
$CaCl_2*2H_2O$ 294.1g

Stock Soution 2
KH_2PO_4 136.1g

Stock Solution 3
$FeC_6H_5O7*H_20$ 4.99g
$MgSO_4$ 87.0g
$MnSO_4*H_2O$ 0.338g

Stock Solution 4
H_3BO_3 0.247g
$ZnSO_4*7H_2O$ 0.288g
$CuSO_4$ *$5H_2O$ 0.100g
$CoSO_4*7H_2O$ 0.056g
$Na_2MoO_2*2H_2O$ 0.048g

Mix each ingredient in each stock solution in 1L water. Heating may be necessary to dissolve, especially Solution 4. Do not mix stock solutions without diluting first, precipitates will form reducing the availability of the nutrients in the final solution.

To make final nutrient solution, mix 1ml of each stock solution in 1L of RO water. Mix thoroughly between each addition to avoid forming any precipitates. The pH may need to be adjusted with Sodium Hydroxide, NaOH, to 6.5. This solution may then be used to water plants.

Yeast Mannitol Agar

1.0 g yeast extract
10.0 g Mannitol
0.50 g Dipotassium Phosphate
0.20 g Magnesium Sulfate
0.10 g Sodium Chloride
1.0 g Calcium Carbonate
15.00 Agar

Mix above ingredients in 1L RO water, add mixing bar and heat to dissolve the agar. Pour 500 ml into each of two 1000ml Erlenmeyer flasks. Stop flask with a ball of cotton held together with cheesecloth. Cover top of flask with aluminum foil. Autoclave for 25 min at 25 psi.

Cool under sterile hood until it is warm to the touch, but still liquid. Pour enough media into each petri dish to cover the bottom. Allow to cool until solid. Media may be used immediately or stored wrapped in the plastic sleeve the petri dishes were packaged in in the refrigerator.

Yeast Mannitol Broth

1.0 g yeast extract
10.0 g Mannitol
0.50 g Dipotassium Phosphate
0.20 g Magnesium Sulfate
0.10 g Sodium Chloride
1.0 g Calcium Carbonate

Mix above ingredients in 1L RO water, add mixing bar and heat to dissolve the agar. Pour 250ml into each of four 500ml Erlenmeyer flasks. Stop flask with a ball of cotton held together with cheesecloth. Cover top of flask with aluminum foil. Autoclave for 25 min at 25 psi.

Cool under sterile hood. Inoculate flask with 1ml of active liquid culture or 1ml rehydrated sample which had been lyophilized for long term storage. Rhizobia may also be used from a culture on solid media by scraping the colony off of the agar and dropping a portion of it into the liquid.

Place flask on an orbital shaker adjusting such that the liquid is gently moving. The culture is ready to use after 3 days.

Rating “0”

No nodules present.

Rating “3”

Moderate nodulation. Pink/orange color indicates nodules are functioning. Nodules concentrated around crown roots.

Rating “6”

Superior nodulation. Pink/orange color indicates nodules are functioning. Nodules diffuse and present not only on crown roots but also lateral roots.