

Isolation of Plant Growth Promoting Bacteria

Some soil bacteria can have a direct or indirect impact on plant growth. Collectively, these bacteria are called PGPRs, plant growth promoting bacteria. Most of these bacteria inhabit the rhizosphere, a region around the plant root where materials released from the plant root promote microbial growth. PGPRs in the rhizosphere can promote plant growth directly by producing products that enhance plant growth or indirectly by inhibiting plant pathogens. A number of PGPRs have been shown to express the enzyme, 1-aminocyclopropane-1-carboxylate deaminase. This enzyme converts 1-aminocyclopropane carboxylate (ACC) to α -ketobutyrate and ammonia. ACC is an intermediate in the biochemical pathway for the production of ethylene in plants. Under stressful conditions, such as increased salt, flooding, drought, toxic metals and pathogens, plants will increase their production of ethylene which has an adverse effect on root elongation. By converting ACC to α -ketobutyrate and ammonia, PGPRs allow for the sustained growth of the roots.

Over the next several of weeks we will attempt to isolate PGPRs and then use standard biochemical test to identify your isolates to the Genus and species.

Cleavage of ACC to α -ketobutyrate and ammonia by ACC deaminase:

Interaction between the plant root and the bacterium:

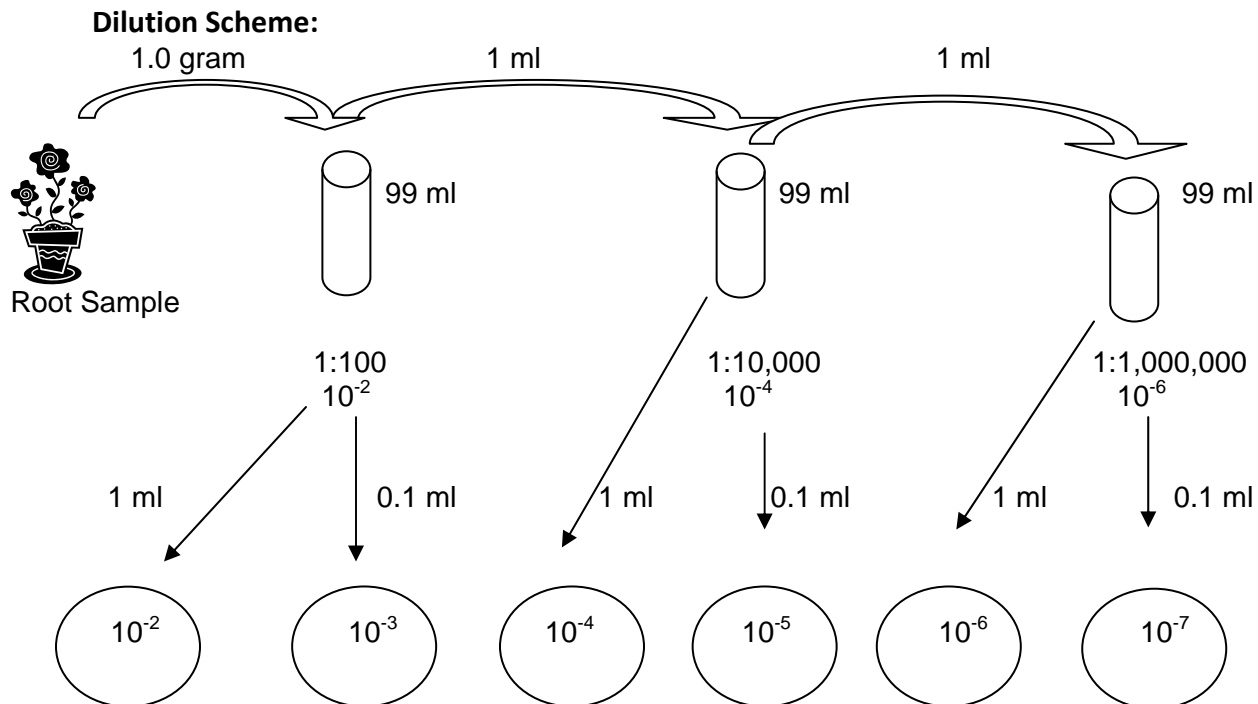
Week 1: Collection of Bacteria from the Rhizosphere

In this portion of the lab we will use selective media and serial dilution to isolate bacteria from plant roots. We will also enumerate the total number of bacteria in the rhizosphere. Please refer to Ex. 22 in your lab manual for the basic protocol on serial dilution and bacterial enumeration. **Please be sure to keep detailed laboratory notes in your notebook!**

Working per bench side:

1. Obtain a plant that has most of the soil shaken off of the roots; what is remaining is the rhizosphere. Be sure to record the type of plant in your notebook.
2. Label 1 bottle containing 99 ml PBS (phosphate buffered saline) 10^{-2}
3. Obtain 2 bottles of 99 ml PBS and label 10^{-4} and 10^{-6}
4. Cut 1.0 gram of roots from the plant and place into the 10^{-2} bottle of PBS. This is a 1:100 or 10^{-2} dilution. Shake the bottle vigorously for at least 30 seconds. A good shake is necessary to dislodge the bacteria.
5. Pipette 1 ml from the 10^{-2} dilution bottle into the 10^{-4} dilution bottle and mix well by shaking.
6. Using a clean pipette tip, pipette 1 ml from the 10^{-4} dilution bottle into the 10^{-6} dilution bottle and mix well.
7. You are now ready to plate the samples. We will plate on to two separate media. TSA, to count total bacteria and M9 with ACC, a minimal media in which ACC is the only nitrogen source so that we select for PGPRs which cleave ACC.
8. Divide the bench side up into teams, one team will plate the TSA plates and the other team will plate the M9 plates.
9. For the TSA plates:
 - a. Label 6 petri dishes: 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} and TSA
 - b. Using the 10^{-2} bottle, pipette 1 ml into the 10^{-2} plate and 0.1 ml (100 ul) into the 10^{-3} plate.
 - c. Using the 10^{-4} bottle, pipette 1 ml into the 10^{-4} plate and 0.1 ml into the 10^{-5} plate.
 - d. Using the 10^{-6} bottle, pipette 1 ml into the 10^{-6} plate and 0.1 ml into the 10^{-7} plate.
 - e. Obtain a bottle of liquefied TSA from the 50°C water bath and pour into the 6 petri dishes. Gently swirl to mix the bacteria into the medium and allow to harden.
 - f. Incubate the plates at 27°C until next lab period.
10. For the M9 w/ACC plates:
 - a. Label 4 petri dishes: 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} and M9.

- b. Using the 10^{-2} bottle, pipette 1 ml into the 10^{-2} plate and 0.1 ml (100 μ l) into the 10^{-3} plate.
- c. Using the 10^{-4} bottle, pipette 1 ml into the 10^{-4} plate and 0.1 ml into the 10^{-5} plate.
- d. Obtain a bottle of liquefied M9 w/ACC and pour into the 4 plates. Gently swirl to mix the bacteria into the medium and allow to harden.
- e. Incubate the plates at 27°C until next lab period.



Week 2: Enumeration of Bacteria and Pure Culture of PGPR

1. Examine both sets of plates for growth.
 - a. TSA Plates: Choose the plate that has between 20 and 200 colonies on it. Count the number of colonies and calculate the number of bacteria in the original root sample (cfu/g). Record in your notebook and on the board. I will then post all results on Blackboard.
 - b. M9 w/ACC: Since this medium selects for bacteria that produce ACC deaminase only PGPRs should grow on this media and these are the ones we are interested in identifying. Again, choose the plate that has between 20 and 200 colonies on it. Count the number of colonies and calculate the number of bacteria in the original root sample (cfu/g). Also, calculate the ratio of PGPR to total bacteria

isolate. Record in your notebook and on the board. I will then post all results on Blackboard.

- c. **INDIVIDUALLY:** Choose a well isolated colony from the M9 plate and streak for isolated colonies using the quadrant streak method (Ex. 10 in your lab manual). Make sure the bottom of the plate is labeled with your name and section number. This is the bacterium you will use for further testing and identification of an unknown bacterium. Please make sure to keep good records of everything you do in your laboratory notebook!
Incubate the plates at 27°C for 48 hours.

Week 3: Test Isolates for Plant Growth Promotion and Start Unknown Identification

Check your plates from last week to make sure you have a pure culture. If not please see me.

1. Testing for plant growth promotion.
 - a. Obtain a small glass beaker, place twenty seeds in the beaker and cover with 1.5 % sodium hypochlorite (bleach). Allow to sit for 15 minutes to surface disinfect the seeds.
 - b. Rinse the seeds well with sterile dH₂O
 - c. Obtain two plastic petri dishes and label one “control” and the other “test”. Place a couple of paper towels into the bottom of the petri dish.
 - d. Moisten the “control” plate paper towels with Farheus solution and place ten seeds on the paper towels and cover.
 - e. Take a large colony or several small colonies from your pure culture plate and place in 5 ml Farheus solution, mix well and then moisten the paper towel in the “test” plate.
 - f. Place ten seeds in the test plate and cover.
 - g. Incubate both plates in the dark at 29°C for 7 days.
2. Unknown Identification.
 - a. Follow the steps outlined in the handout and lab manual for the next 6 weeks.
 - b. Identify your bacterium to the Genus and species.

Week 4: Finish Plant Growth Promotion

Obtain your seeds from last week and measure the length of the roots in both the control and test plates. Make a table in you notebook to record your results. Take the average for each set of seeds and record your results on the board. Did your bacterium promote root growth?

$$\% \text{ growth promotion} = \frac{\text{test-control}}{\text{control}} \times 100$$

Recipes

M9 media:

- A. 5x M9 minus NH_4Cl_2 , per liter: 33.9 g Na_2PO_4 , 15 g $\text{K H}_2\text{PO}_4$, 2.5 g NaCl. Autoclave
- B. 20 % glucose, filter sterilize. (2%, final conc.)
- C. 1.0 M MgSO_4 . Autoclave
- D. 1.0 M CaCl_2 . Autoclave
- E. 25X ACC (sigma) 0.75 g in 100 ml dH_2O , filter sterilize (Final conc. 3 mM)
- F. 15 g/L agar. Autoclave

Autoclave 4.5 g agar in 200 ml dH_2O in a 500 ml media bottle. Allow all components to cool to 50°C and then add to agar 60 ml 5x M9, 30 ml glucose, 0.4 ml MgSO_4 , 0.04 ml CaCl_2 .

Day of use, melt M9 in the microwave, cool to 50°C and add 12 ml ACC stock just before use.

Fahraeus Solution:

Per liter: 0.1 g CaCl_2 , 0.12 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g KH_2PO_4 , 0.15 g Na_2HPO_4 , 0.005 g FeCitrate, traces of Mn, Cu, Zn, B, Mo.

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Sample Results:

Fall 2010

TUES AM

Group	Plant	TSA cfu/ml	M9/ACC cfu/ml	% PGPR
1	Small tobacco, planter	4.8×10^5	1.2×10^5	25
2	Small tobacco, planter	6.3×10^5	3.1×10^5	49
3	Large tobacco, planter	1.2×10^6	6.0×10^5	50
4	Marigold, planter	1.09×10^6	1.5×10^5	13.7
5	Small tobacco, planter	1.94×10^5	1.63×10^5	84
6	Large tobacco, planter	1.58×10^5	8.7×10^4	55

TUES PM

Group	Plant	TSA cfu/ml	M9/ACC cfu/ml	% PGPR
1	Large tobacco, planter	8.2×10^5	1.08×10^6	131.7
2	Large tobacco, planter	6.5×10^5	1.64×10^6	252.31
3	Small tobacco, planter	3.6×10^6	4.0×10^6	111.1
4	Small tobacco, planter	5.1×10^6	7.0×10^6	137.25
5	Marigold, planter	7.8×10^6	5.7×10^5	73
6	Large tobacco, planter	2.34×10^5	1.77×10^4	7.5

TUES PM

Student	Gp	plant	seed	wk	control	Test	%increase	notes
Neuman	1	lg. tobacco	oats	1	8.17 mm	10.5 mm	28.50%	1 test germinated, 3 control germinated
Kauffman	2	lg. tobacco	oats	1	4.9 mm	4.2mm	0%	4 test germinated, 4 control germinated
Luckey	2	lg. tobacco	oats	1	6.8 mm	7.2 mm	5.88%	all test germinated, 4 control germinated
Olsen	3	sm. Tobacco	oats	1	9.3mm	6.5 mm	0.00%	1 test germinated, 4 control germinated
Bloom	5	Marigold	Oats	1	84.25mm	28.75mm	0%	4 test germinated, 4 control germinated
Furman	3	Sm. Tobacco	Oats	1	7.075mm	6.1mm	0.00%	1 test germinated, 4 control germinated
Dickinson	3	sm. Tobacco	oats	1	9.42mm	8.175mm	0%	4 test germinated, 5 control germinated
Bakerink	2	lg. tobacco	oats	1	70.0 mm	30.0 mm	0.00%	all test germinated, 4 control germinated
Moore	5	Marigold	corn	1	no growth	1.6 mm	0.00%	control didn't germinate, 3 test germinated
Fuentes	2	lg. tobacco	oats	1	3.975mm	6.6mm	66.04%	4 control germinated, 2 test germinated
Mills	4	sm. Tobacco	corn	1	31mm	34.7mm	12%	2 control and 3 test seeds germinated
Gasser	4	sm. Tobacco	corn	1	2.4mm	2.5mm	10%	1 test and 4 control seeds germinated
Salem	6	lg. tobacco	Corn	1	3.82mm	3.8mm	0.00%	4 test and 1 control seed germinated
Vittese	1	lg. tobacco	corn	1	15.6mm	0.1mm	99%	3 control and 1 test seed germinated
Smith	1	lg. tobacco	corn	1	8 mm	19mm	137.50%	2 control and 4 test germinated
Meadow	6	lg tobacco	corn	1	2.8mm	33.8mm	1107%	only 3 tests and 3 controls germinated
Moore	4	sm. Tobacco	corn	1	34.6mm	53.25mm	53.90%	3 control germinated and 4 tests germinated

Student	Gp	plant	seed	wk	control	Test	%increase	notes
Moore	5	Marigold	corn	2	5.1 mm	1.6 mm	0%	2 test didn't germinate, 5 control germinate
Gasser	4	Sm. Tobacco	corn	2	12.3mm	8.1mm	0%	3 test didn't germinate, 5 control germinate
Salem	6	Lg. Tobacco	corn	2	16.7mm	43.3mm	159.40%	Bacteria are growth promoting
Meadow	6	Lg Tobacco	corn	2	85.8mm	404mm	371%	Bacteria are growth promoting
Smith	1	Lg Tobacco	corn	2	20mm	39.4mm	97%	3 control didn't germinate.
Vittese	1	Lg. Tobacco	corn	2	107.6mm	11.0mm	89.80%	4 control and 1 test germinated