Seed-bag experiment

Arnold Lab, University of Arizona
Contact arnold@ag.arizona.edu before use in publication
This version: Ming-min Lee and Betsy Arnold, November 2016

Goals of this project: To collect seed-associated microbes from arid agricultural and unimproved soils in Arizona; to catalog these microbes, to determine their effects on seed germination, and to analyze site metadata to discover any patterns in positive and negative microbe-seed associations.

I. Seed Bag Construction

Purpose: To construct sterile seed bags for seed burial. Bags allow soil and microbes to contact seeds while excluding insects and other arthropods that might damage seeds.

Number of bags: 18 bags per plot are required, along with two bags to be used as field controls and an additional two as lab controls. We typically have 2 or 3 plots per site.

Materials:

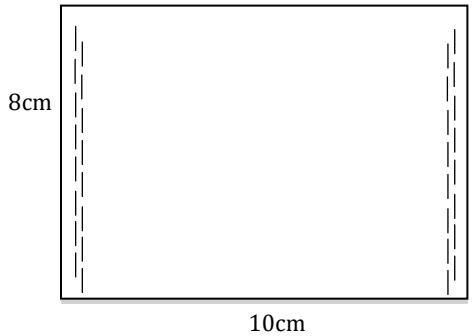
- 50µm polyester filter material (https://www.dudadiesel.com/search.php?query=%2Bpolyester+%2Bsheet)
- Scissors
- Rulers
- Staplers and staples
- Aluminum foil
- Laminar flow hood or alcohol burner
- Forceps, spoonulas, teaspoons etc.
- Infrared or bead sterilizer, or alcohol burner and ETOH for sterilizing tools
- Autoclave
- Seeds

1. Seed bags are constructed from 50µm polyester filter material cut into ~16cm x 10cm rectangles, folded in half and stapled with staggered rows. (Leave the top open until filled with seed.)

- Staggering staples closes spaces that might allow seed predators to enter the bag

Correct: Incorrect:

Final bag should be approximately 8cm x 10cm



2. Wrap bags securely in foil and sterilize by autoclaving no more than 24 hours prior to filling with seed.

3. Fill bags under the hood or using sterile technique around an alcohol burner. Each bag receives 5 bean, 5 corn and 40 wheat seeds using sterilized implements. If native seeds or surface sterile seeds are to be used, see part IV, 4 (steps 2 & 3) for surface sterilization procedure.

4. Staple bags closed and re-wrap in foil to maintain sterility before removing from hood or moving away from alcohol burner. Place rewrapped packets in clean container or Ziploc bags for transport. Lab control bags are to stay in the lab.

II. Burial

Purpose: To deploy seed bags at the chosen field site and to collect site metadata and field soils for analysis.

Materials:

- Measuring tape
- Pin flags
- Hand trowels and/or shovels
- Quart size Ziploc bags
- Sharpie
- GPS

1. In your site(s), delimit a 2 m X 1 m plot with pin flags and bury 18 bags in shallow holes (approximately 5 cm., but deep enough to cover bags completely with soil).

2. From each corner of each plot, remove the top 1 cm of soil and any rocks or debris. Then collect ca. 1 liter of soil for analysis (place in Ziploc bag). Label each bag with site name and a unique number, 1 through 4, along with the date and names of collectors. Ex. “Mesquite-3 Sarah Chen 8 Nov. 2016”

3. Collect metadata using the provided datasheet.

III. Soil Processing

Purpose: To process and ship collected field soil for analysis by an independent laboratory (Motzz laboratory), requiring 300 grams of dry sifted soil per sample.

Materials

- No. 10 sieve (mesh size 2mm)
- Trays
- Container for sifted soil
- 70% ETOH
- Scale
- Quart size Ziploc bags
- Sharpie
- Paper towels
- Drying oven

1. If soils are wet, dry them in a drying oven or spread in a thin layer (<2 cm) to air dry in clean, appropriately labeled trays or weigh boats. Set aside sufficient material to achieve a final dry weight of ~315g sifted material.

2. Once soils are dry, pass through sieve and weigh. Discard large, unsifted particles. Work with samples from one site at a time, being careful to label throughout and to remove all material from previous sample before processing a new one. Wipe container and sieve with ETOH between sites. If soils are still moist after sieving, place in drying oven or continue to air dry and reweigh.

3. Transfer ~315 g. of this processed soil to appropriately labeled Ziploc bags.

4. Soil is now ready to be mailed to laboratory for testing.

IV. Retrieval and Processing

Purpose: To retrieve previously buried seeds, surface sterilize and plate them under axenic conditions to isolate seed associated microbiota.

Materials

- 50ml centrifuge tubes
- Forceps, teaspoons, spoonulas or other implements
- ETOH burner
- 95% ETOH
- 0.5% Sodium Hypochlorite (from household bleach), made fresh
- 70% ETOH
- Clean 100mm Petri dishes
- Laminar flow hood
- 150mm 2% MEA Petri dishes
- Infrared or bead sterilizer, or flame and ETOH for sterilizing tools
- Parafilm
- Sharpies

1. After ~10 days, retrieve all bags and pin flags, remembering to return site(s) to as natural a state as possible

2. After arriving at the lab, carefully open each bag with scissors and remove all seeds from each bag into a corresponding sterile 50mL centrifuge tube with clean implements.

→ If you have 18 bags/site, you will then have 18 tubes per site, each containing the three seed types from their respective bags.

3. Take notes on any changes to the seed contents such as germination or fungal growth in 1.) your laboratory notebook 2.) Project datasheet for the site (will be provided). Remember to specify site and bag number. Below is an example of how these notes may be organized in your notebook:

Date processed: 14 XI 2016			Your Name: Joe Smith		
Site	Bag #	#C germ.	#S germ.	#W germ.	Comments
CAC-Cotton	1	1	0	10	Wheat seeds with primary leaves
CAC-Cotton	2	1	2	15	White fungus on soy seeds
CAC-Cotton	3	0	0	3	Clean seeds

4. Surface sterilize seeds:

• Empty contents of single tube into a petri dish, labeling dish with the respective bag #

• Fill with sterilant (as below) and swish vigorously with the lid in place. After the specified time, drain the liquid out without opening the dish. When refilling dish between treatments, open the lid just enough to refill.

95% ETOH for 10 s.

0.5% Sodium hypochlorite for 2 min.

70% ETOH for 2 min.

Remove liquid by tipping dishes to the side and draining sterilant into the sink with the lid in place. This point is especially important after the 70% ETOH wash. Transfer closed plates to laminar flow hood or other sterile work environment.

→ Take care not to lose any seeds when draining sterilant

→ Sharpie is easily erased by ETOH, be mindful and relabel if necessary, or overlay with clear tape to protect labeling

5. Under sterile conditions, tip open the lid and allow seeds to dry slightly (but not entirely) before arranging them on 150mm 2% MEA plates with sterile implements.

→ Arrange seeds to maximize distance between them on the plate.

6. Label plates with **site** and **bag number**, **date**, and your **initials**. Seal plates with a double layer of Parafilm and allow to germinate at room temperature in the dark for ~four days.

→ Use caution when moving plates as seeds may roll.

V. Scoring and Isolation.

Purpose: to quantify presence of microbial growth on seeds; to obtain pure cultures, then identity them using molecular biology.

Materials

- Laminar flow hood or alcohol burner
- Sterile toothpicks
- 35mm plates with 2% MEA
- Parafilm
- Sharpie

1. After incubation period, score plated seeds by observing and recording the following for each replicate in each site:

How many of each type have germinated? What are their traits
How many of each seed type exhibit signs of fungal infection?
How many of each seed type exhibit signs of bacterial infection?
Are there any coinfections?

2. Once microbes have been identified and recorded, each one is recorded with an unique isolate number and transferred (in a sterile environment) onto new media to be grown in pure culture.

3. When cultures have sufficient, axenic growth, duplicate water vouchers and a DNA voucher are created.

→ Water vouchers are for long term storage of tissue materials that may be regrown.

→ DNA vouchers will be used in PCR of the ITS region for sample identification.