PROTOCOL FOR ESTABLISHING AXENIC CULTURES

1. To avoid contamination, always carry out the procedures under the laminar flow hood at eye level, not too close to the hood opening.

2. Before beginning, thoroughly clean the table surface of the laminar flow hood twice with spray 95% EtOH.

3. Take the required number of Gerber/Hatcher's agar culture jars (4 per capsule) from the refrigerator, liberally spray the outer surface of each jar twice with 95% EtOH and then place in the hood.

4. Place all syracuse dishes, with sporophytes, in the outer right corner of the hood along with a single, disposable plastic dropper.

5. Wash a 500 ml beaker twice in 95% EtOH and place in the center of the hood. Also, place a twice washed (95% EtOH), 10 ml pipetter on top of the beaker.

6. Prepare the sterilizing solution (using a plastic graduated cylinder) by placing 15 ml of Clorox in 85 ml of distilled water with 1-2 drops of Tween. Thoroughly mix the solution by placing one hand over the mouth of the cylinder and inverting the tube at least 5 times. **Always make a fresh solution and discard any leftovers** since Clorox will lose its effectiveness in a very short time.

7. Before beginning the transfer procedure, put on latex gloves and spray them twice with 95% EtOH.

8. Set the timer for 2 minutes and nearly fill an empty, sterilized glass test-tube (screw-top cap) with the Clorox solution.

9. Use the disposable, plastic dropper to transfer the capsule to the Clorox tube, replace the screw-cap and start the timer. Gently agitate the solution being sure that the capsule sinks to the bottom of the tube.

10. When the timer shows 30 seconds remaining, begin to **slowly** decant the solution into the 500 ml beaker being careful not to lose the capsule.

11. Use a sterile pipette with the pipetter to remove the remaining solution (take care not to touch the pipette to anything in the hood. After removing all of the Clorox solution, pour 1/2 tube of sterilized water over the capsule.

12. Leave the capsule in this rinse for 2 minutes; decant, using another sterile pipette to remove the residual water. Repeat this rinsing process a second time.

13. After removal of the last sterile water wash, pipette 5 ml of sterile water into the tube and use one of the sterile, wrapped grinding rods to rupture the capsule and disperse the spore mass.

14. Use a new sterile pipette to transfer the spore suspension to four replicate culture jars (roughly 20-30 drops of spore suspension per jar). Always complete the operation holding the jar at eye level and tilt the cap open enough to place the tip of the pipette over the agar without touching any surfaces. **Never completely remove the lid.**

15. Once all operations are complete, label all jars and place in the appropriate growth chamber. Dispose of the Clorox pipette in the glass discard box and place the rinse pipettes in the appropriate basket for autoclaving.

16. Monitor weekly for germination/contamination. Once germination is seen, place the vermiculite "backup" plants in a minipack labeled "vermiculite" (with the harvest date) and place in the voucher packet.

PROTOCOL FOR TRANSFERRING AXENIC CULTURES

1. Turn on the bacto-incinerator 30 minutes before beginning transfer procedures.

2. Rinse the transfer hood and culture jars with 95% EtOH. Put on latex gloves and spray twice with 95% EtOH.

3. Loosen the lids of the stock cultures from which the transfers are to be made and the new culture jars.

4. Once the bacto-incinerator reaches operating temperature, flame the transfer needle (at least 15 seconds) **being sure to heat the entire length of the wire**. Once heated, remove and cool (in the hood) until no red color is seen and place into the container of 95% EtOH under the hood. Again, be sure that the entire surface of the wire is in the EtOH solution. Remove the needle from the EtOH and allow the alcohol to evaporate, then reflame the needle.

5. First, touch the hot needle to the agar of the stock culture to cool it and then transfer a small amount of the plant material from the stock culture to a new culture jar.

6. Between each transfer, flame and cool the transfer needle twice to ensure sterility. Always place the needle in the 95% EtOH when not in use.

7. When finished, label each new culture with all pertinent data from the stock culture. Indicate the current date of transfer at the bottom of the label.