2. Cultivation

2.1 Inoculation from spores (originally written by Yuji Hiwatashi, and edited by Mitsuyasu Hasebe: last updated 16 June, 2004)

Thousands of spores are contained in a sporangium. After a sporophyte with a sporangium is sterilized, the sporophyte is cracked in distilled water and spores are dissolved. Calcium ion is necessary for spore germination, and do not forget to put 5 to 10 mM Ca2+. It is better to seal a petri dish with “surgical tape” for long cultivation (more than a week). Do not use parafilm for better aeration.

2.1.1 preparation

1. Spore Germination Medium（BCD+10mM Ca+ 5mM Ammonium tartrate）：1000ml

H_2O	900 ml
stock solution B	10 ml
stock solution C	10 ml
stock solution D	10 ml
Alternative TES	1 ml
500 mM ammonium tartrate	10 ml（final conc.＝ 5 mM）
$CaCl_2$・$2H_2O$	1.5 g（final conc.＝10 mM）
agar（Sigma; A6924）	8 g（final conc.＝0.8%）
H_2O	fill up to 1000ml

→autoclave, pour to 90 mm petri dishes. After agar was solidified, open a lid, keep drying for 30 min at r.t. in cleanbench. These plates are preserved at r.t.

2. Sterilized water

3. Cut cellophanes to a little bit smaller than the size of the plate (the same one as used for E. coli culture). A circle cutter is useful to cut cellophane. Dip in 5 mM EDTA (pH 8.0) in a glass plate, then autoclave (120C 20 min). You can put more than 30 sheets

of cellophanes in a plate. After autoclaving, wash with MilliQ water for several times to remove EDTA. Dip cellophane in MilliQ water, then autoclave (120C 20 min). These cellophane seats are used to overlay on agar media and prevent protonemata from growing into the media.

Some cellophane prohibits Physco growth. You should compare the growth with or without cellophane.

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4. Two tweezers, yellow tips and blue tips for pipetman→autoclave

5. 10% Sodium Hypocholorite Solution (Antiformin) : NOT necessary to autoclave.

2.2 procedure

You should perform the following steps in a cleanbench.

1. Overlay agar medium with a sheet of cellophane. Not to include air between media and cellophane.

2. Put one or two sporangium in a 1.5ml microtube, then pour 1 ml of 10% Antiformin to sterilize. One sporangium contains >1000 spores. It may be better to see the sporangium by microscope or magnifying glass to confirm it is not empty.

3. Mix gently for 5min and discard supernatant using a pipetman. Usually the sporangium sink at the bottom of tube, but be careful not to crack the sporangium until step 7. Otherwise, tiny spores are dispersed in a tube and it is not possible to recover. Spores may not be viable in an antiformin.

4. Add 1ml sterilized water into the tube and mix gently for few minutes.

5. Discard supernatant with a pipetman.

6. Repeat step 5 and 6 at four times.

7. Add 1ml sterilized water, crush the sporangium with the tip of a yellow-tip, and mix gently. When you crush the sporangium, you can see spores disperse in the tube.

8. Pour 0.2ml the spore-solution at the center of the agar medium overlayed with a cellophane.

9. Add 1ml sterilized water to the drop of the spore-solution on the agar medium. Spread the spore solution over the agar medium by gently shaking the plate.

10. Incubate at 25C under continuous light.

11. Spores will germinate in a couple of days and protonemate start to grow. After about 15 days, gametophores start to grow.