

## 2. Cultivation

### 2.2 Cultivation of protonemata and gametophores (originally written by Yuji Hiwatashi, and edited by Mitsuyasu Hasebe: last updated 16 June, 2004)

**BCDATG medium** is used for regular cultivation. Protonemata grow faster in BCDATG medium than in other media, and is good to collect materials for nucleic acid extraction in relatively short period. However, senescence of plants is a bit enhanced in BCDATG, and BCDATG should not be used for long culture. Another good point of BCDATG is easiness to detect contamination because of glucose in the medium.

**BCDAT medium** is mostly similar to BCDAT, but growth of protonemata is a bit slower. We use BCDAT for selection with G418 and/or hygromycin after transformation, because of its long selection period. Hard to detect contamination (contaminants also grow slower), and should be careful.

Protonemata grow slower in BCD + 1 mM Ca<sup>2+</sup> medium (often called just **BCD medium**), but differentiation of chloronemata and caulonemata is more easily observed in BCD medium than other media. Furthermore, irregular cell divisions at young stages of bud development observed in other media are not observed in BCD medium, and should be good for bud observation.

#### 2.2.1 stock solution

store at 4C. Stock solution D is easily oxidized, and should be used in 2 to 3 months.

stock solution A ( × 1 0 0 )

Ca(NO <sub>3</sub> ) <sub>2</sub> · 4H <sub>2</sub> O	118 g (0.5 M)
FeSO <sub>4</sub> · 7H <sub>2</sub> O	1.25 g (4.5 mM)
	up to 1000 ml with H <sub>2</sub> O

stock solution B ( × 1 0 0 )

MgSO <sub>4</sub> · 7H <sub>2</sub> O	25 g (0.1 mM)
	up to 1000 ml with H <sub>2</sub> O

stock solution C ( × 1 0 0 )

KH <sub>2</sub> PO <sub>4</sub>	25 g (1.84 mM)
	adjust pH to 6.5 with 4M KOH
	up to 1000 ml with H <sub>2</sub> O

stock solution D ( × 1 0 0 )

KNO <sub>3</sub>	101 g (1 M)
FeSO <sub>4</sub> · 7H <sub>2</sub> O	1.25 g (4.5 mM)
	up to 1000 ml with H <sub>2</sub> O

Alternative TES ( × 1 0 0 0 )

CuSO <sub>4</sub> · 5H <sub>2</sub> O	55 mg (0.22 mM)
H <sub>3</sub> BO <sub>3</sub>	614 mg (10 mM)
CoCl <sub>2</sub> · 6H <sub>2</sub> O	55 mg (0.23 mM)
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	25 mg (0.1 mM)
ZnSO <sub>4</sub> · 7H <sub>2</sub> O	55 mg (0.19 mM)
MnCl <sub>2</sub> · 4H <sub>2</sub> O	389 mg (2 mM)
KI	28 mg (0.17 mM)
	up to 1000 ml with H <sub>2</sub> O

500mM Ammonium Tartrate ( × 1 0 0 )

Ammonium Tartrate	92.05 g
	up to 1000 ml with H <sub>2</sub> O

50mM CaCl<sub>2</sub> ( × 5 0 )

CaCl <sub>2</sub> · 2H <sub>2</sub> O	7.35 g
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	up to 1000 ml with H <sub>2</sub> O
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autoclave

### 2.2.2 regular media

#### **BCD + 1 mM Ca medium (BCD medium): 1000 ml**

H <sub>2</sub> O	900 ml
stock solution B	10 ml
stock solution C	10 ml
stock solution D	10 ml
Alternative TES	1 ml
50mM CaCl <sub>2</sub> · 2H <sub>2</sub> O ( if use powder )	20 ml ( = 1 mM ) ( 0.15 g )
agar ( Sigma A6924 )	8 g ( = 0.8% )
	up to 1000 ml with H <sub>2</sub> O

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.

#### **BCDAT medium: BCD + 1 mM Ca + 5 mM ammonium tartrate medium: 1000 ml**

H <sub>2</sub> O	900 ml
stock solution B	10 ml
stock solution C	10 ml
stock solution D	10 ml
Alternative TES	1 ml
500mM ammonium tartrate.	10 ml ( final conc. = 5 mM )
50mM CaCl <sub>2</sub> · 2H <sub>2</sub> O ( as powder )	20 ml ( final conc. = 1 mM ) ( 0.15 g )
agar ( Sigma A6924 )	8 g ( final conc. = 0.8% )

up to 1000 ml with H <sub>2</sub> O
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**BCDATG medium: BCD + 1 mM Ca + 5 mM ammonium tartrate + 0.5% glucose medium: 1000 ml**

Add 5 g glucose to 1000 ml BCD medium

### **2.2.3 special reagents**

Thiamine chloride (M.W. 337.3)

0.5 mg par 1000 ml medium (final conc. 1.5 uM)

p-amino benzene (M.W. 137.1)

247 ug par 1000 ml medium (final conc. 1.8 uM)

### **2.2.4 cultivation conditions**

#### **Light:**

We usually use continuous light (40 umol photons m<sup>-2</sup>s<sup>-1</sup>) conditions or 16 light and 8 hours dark conditions. When you prefer to induce gametangia, short day conditions are better (see 2.3).

#### **Temperature:**

25C for regular cultivation.

15C for gametangia induction.

### **2.2.5 vegetative propagation of protonemata**

You can preserve protonemata at 4C under dark or low light conditions for long period.

To prevent drying up, seal a petri dish with parafilm.

To vegetative propagation of protonemata, use young protonemata cultivated for 5 to 7 days.

## **Preparation**

1. BCDAT or BCDATG medium
2. distilled water
3. cellophane

### **pretreatment of cellophane**

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

- a. 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 cellophan.
  - b. 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.
  - c. After autoclaving, wash with MilliQ water for several times to remove EDTA.
  - d. Dip cellophane in MilliQ water, then autoclave (120C 20 min).
  - e. These cellophane seats are used to overlay on agar media and prevent protonemata from growing into the media.
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4. Polytron: we usually use model PT2100 or MODEL K with DA2121/2 shaft. You can use motor and pestle instead of polytron.

### **Procedures (all procedures should be done in cleanbench)**

1. put a seat of cellophane on each medium.
2. Pour 20 ml distilled water in a glass test tube.
3. Remove protonemata from culture dish using forceps, and move to the glass test tube.

4. Digest with “polytron PT2100” at minimum power (in case of “MODEL K power 4 to 5) for 10 sec.
5. Pour 2 ml digested green solution on a cellophane-covered plate with pipet.
6. Cultivate at 25C.