Standard Operating Procedure for Hazardous Chemicals

Principal Investigators: Chung-Jui Tsai and Scott A. Harding
Building and rooms: Davison Life Sciences Building, Lab B310

Chemical(s)	MS (Murashige and Skoog) Basal Salt, Vitamin solution, MES hydrate, L-glutamine, sucrose, 2iP (N^6-2-isopentenyladenine), BAP (6-benzylaminopurine), IBA (indole-3-butyric acid), NAA (α-naphthaleneacetic acid), TDZ (thidiazuron), Acetosyringone in DMSO, Basta, Claforan (Cefotaxime sodium salt), galactose, Hygromycin, Kanamycin, Timentin, Bleach, DMSO (Dimethyl sulfoxide)
Process	**Transformation of *Populus tremula* x *P. alba* INRA 717-1B4**
Specific Hazards *referred to MSDSs for more detailed information*	DMSO (Dimethyl sulfoxide): Embryotoxin. Irritant. Bleach: Corrosive.
Personal protective equipment	Wear 3-5 mil nitrile gloves when handling (concentrated) Bleach and DMSO. Chemical safety goggles and lab coat should be worn when splash potential exist
Engineering/ventilation controls	Emergency shower and eyewash accessible. DMSO: chemical fume hood (when working with large quantities)
Special handling procedures and storage requirements	Store DMSO in general storage area above the electrophoresis bench in Rm B310, away from oxidizers, acids, and flammable reagents. Store MS, Vitamin @ 4°. Store PGH, antibiotics @ -20°C. Store bleach under the sink.
Spill and accident procedures	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. Eye exposure: Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
for hazardous chemicals only	Small (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 2L): Evacuate the room, notify PIs and call 2-5801 to request emergency spill assistance from the Environmental Safety Division.
Waste disposal	Bleach <10% can be disposed of down the drain with large quantity of water. DMSO wastes must be collected and labeled as hazardous waste according to the SOP for Hazardous Waste Disposal.
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: C-J Tsai, 4/24/09
Reviewed by	Name/date: Kate Tay, 8/13/09

Standard Operating Procedure for Transformation of *Populus tremula* x *P. alba* INRA 717-1B4

Chemicals

Culture media:
MS (Murashige and Skoog) Basal Salt Mixture (Caisson MSP01-50LT, formally MSPC0130)
Vitamin solution, 1000X (Caisson MVL01-100ML, formally MVLC0140), already containing *myo*-inositol
MES (Sigma M2933-100g)
L-glutamine (Caisson G010, 100g)
Sucrose

Plant growth regulators and stock concentrations:
2iP (N^6-2-isopentenyladenine, Caisson D006-1g), 5 mM (= 1mg/mL)
BA (BAP, 6-benzylaminopurine, Caisson B001-25g), 0.5 mg/mL
IBA (indole-3-butyric acid, Caisson I002-25g), 1 mg/mL
NAA (α-naphthaleneacetic acid, Caisson N001-25g), 10 mM
TDZ (thidiazuron), 25 mg/mL and 1 mg/mL

PGR stock preparation:
BA, IBA, NAA, zeatin, 2iP, kinetin: *dissolve in 1N KOH or NaOH - do so* ***drop-by-drop*** *with swirling until the powder dissolves (e.g., with minimum volume of alkali). Add ddH_2O to final volume.*

2,4-D and IAA: *use ethanol to dissolve as above (minimum vol), and dilute to final volume with ddH_2O.*

TDZ (Dropp, *50% TDZ by wt****):*** *Prepare concentrated stock at 25 mg/mL in DMSO (e.g., 500 mg Dropp in 10 mL DMSO), mix well and spin to . Store at -20°C. Dilute to 1 mg/mL working stock with 50% DMSO, and store at 4 °C.*

Antobiotics and other selecting agents:
Acetosyringone (4''-hydroxy-3'',5''-dimethoxyacetophenone, VWR TCD2666-005G), 10 mM (1.96 mg/mL) in DMSO
Basta (VWR 100517-880, 1 mg/mL), need to re-do a killing curve
Claforan (Cefotaxime sodium salt, Cassion C032-20g), 300 mg/mL in ddH_2O, filter-sterilized
D(+)-galactose
Hygromycin (PhytoTechnology H385, 100 mg/mL), 50 mg/mL and 5 mg/mL
Kanamycin, 100 mg/mL
Timentin, 200 mg/mL in autoclaved ddH_2O (prepared in the laminar flow)

Additional items needed:

Bleach
Autoclaved ddH_2O
Autoclaved beaker (800 mL), 1
Autoclaved Erlenmeyer flasks (125 mL), 1 per construct
Autoclaved paper towel cut to half length-wise, wrap in aluminum foil.

Plant media preparation:

Add the necessary components to ddH_2O (half of the final media volume) on a stirrer to dissolve, **one by one**. If MES is used in a media, add it last ***after*** both the pH and the volume has been adjusted to the desired. *This is because MES is a buffering agent with a pH range of 5.5-6.7.* If you add MES too early in the preparation, you will end up needing to add more KOH/NaOH/HCl to adjust the pH to the desired pH 5.8.

For solution/media preparation, you should not combine dry powders or concentrated stocks to an empty beaker without first dissolving/diluting them in water. This may cause unexpected chemical reactions or precipitation.

All media should be autoclaved at 120°C for 20 min (**or as appropriate**). Vitamins, certain growth regulators, and antibiotics are filter-sterilized and added to the media after autoclaving. Vitamins may also be added prior to autoclaving. Place the media bottle on a stirrer and *cool until the bottle can be touched with the **inside of your forearm** for several seconds* (less than 60°C). It's **TOO HOT** if your bare hand cannot stand the heat.

ALWAYS double check stock concentrations and adjust if necessary.
DO NOT WASTE. Media containing antibiotics should not be stored for > 1 mo.

CIM (Callus Induction Medium), pH 5.8

To a beaker with ~500 mL of ddH_2O, add the following (**per liter**),

MS Basal Salts	4.33 g
Sucrose	30 g
Vitamin solution	1 ml (if precipitation is observed, warm to room temp to dissolve)
L-glutamine	0.2 g
NAA (10 µM final)	1 ml of 10 mM NAA stock
Bring volume to 1 L, adjust pH to 5.8	
MES	0.25 g
Check/confirm pH to be 5.8	
Gellan gum	3 g
Autoclave	
2ip (5 µM final)	1 ml of 5 mM 2ip stock **(add after autoclave)**
Antibiotics as needed	
Timentin (200 mg/ml stock)	1 ml
Claforan (300 mg/ml stock)	1 ml

Kanamycin at 100 mg/L (final conc); Hygromycn at 10 mg/L (final conc)

SIM (Shoot Induction Medium)

To a beaker with ~500 mL of ddH_2O, add the following (**per liter**)

MS Basal Salts	4.33 g
Sucrose	30 g
Vitamin solution	1 ml (if precipitation is observed, warm to room temp to dissolve)
L-glutamine	0.2 g
TDZ (0.05 mg/L final)	50 µl of 1 mg/mL TDZ
Bring volume to 1 L, adjust pH to 5.8	
MES	0.25 g
Check/confirm pH to be 5.8	
Gellan gum	3 g
Autoclave	
Antibiotics as needed	
Timentin (200 mg/ml stock)	1 ml
Claforan (300 mg/ml stock)	1 ml

Kanamycin at 100 mg/L (final conc); Hygromycn at 10 mg/L (final conc)

SEM (Shoot Elongation Medium)
To a beaker with ~500 mL of ddH_2O, add the following (**per liter**)

MS Basal Salts	4.33 g
Sucrose	30 g
L-glutamine	0.2 g
Vitamin solution	1 ml (if precipitation is observed, warm to room temp to dissolve)
BAP (0.05 mg/L)	100 µl of 0.5 mg/L BAP

Bring volume to 1 L, adjust pH to 5.8

MES	0.25 g

Check/confirm pH to be 5.8

Gellan gum	3 g

Autoclave

Antibiotics as needed	
Timentin (200 mg/ml stock)	1 ml

Claforan can probably be omitted at this point. Keep if Agrobacterium regrowth is observed
Kanamycin at 100 mg/L (final conc); Hygromycn at 10 mg/L (final conc)

RM (Rooting Medium)
To a beaker with ~500 mL of ddH_2O, add the following (**per liter**)

½ MS Basal Salts	2.15 g
Sucrose	20 g
L-glutamine	0.2 g
Vitamin solution	1 ml (if precipitation is observed, warm to room temp to dissolve)
IBA (0.1 mg/L)	100 µl of 1 mg/L IBA

Bring volume to 1 L, adjust pH to 5.8

MES	0.25 g

Check/confirm pH to be 5.8

Gellan gum	3 g

Autoclave

Antibiotics as needed	
Timentin (200 mg/ml stock)	0.5 ml

Kanamycin at 50 mg/L (final conc); Hygromycn at 5 mg/L (final conc)

Bacterial media for Agrobacterium induction

LB (pH 5.4), liquid
Acetosyringone to 20 µM (from 10 mM stock in DMSO), **add before use**
Antibiotics as appropriate, **add before use**

Transformation Protocols

Day 1 Streak *Agrobacterium tumefaciens* on a LB plate containing appropriate antibiotics. Invert and incubate the plate at 28°C for 2 days. **Think about your control! An empty vector or GUS/GFP control would be ideal.**

Day 3 Pick one isolated colony to inoculate 5 mL of LB (pH 7) with antibiotics and grow overnight at 28°C with shaking (250 rpm).

Day 4 Subculture the agrobacteria by diluting 1:100 into 5 mL of LB (pH 5.4) containing suitable antibiotics and **20 µM AS** (in DMSO). Grow overnight at 28°C with shaking. You may want to do a couple of tubes as backup. This step may be skipped.

Between Day 1 to 4 Prepare CIM, with and without antibiotics.

Day 5 Excise young leaves (LPI3-5) from growth chamber plants and surface sterilized them in 10% (v/v) bleach with a few drops of Tween-20 for 10 min, followed by three rinses in sterile water for 10 min each. Cut the leaves into 7 mm squares along the midrib with 5 wounds on the rib. Place leaf disks on CIM media, with the midrib-side facing up. **Do ~30 disks per construct.**

Add *Agrobacterium* directly onto the disks (along the edges and midribs) on CIM (***no antibiotics***) plates using a sterile plastic transfer pipet or a 1 mL pipet. Remove excess agrobacteria. Seal the plates with paraffin and co-cultivate for 2 days in the dark.

Day 7 Carefully transfer leaf disks to a 125-mL flask containing ~50 mL sterile water. Rinse explants with sterile water several times to remove most of the agrobacteria. Damaged leaf disks from previous handling can be eliminated at this point.

Wash in 30 mL ddH_2O with 200 mg/L (20µl of [300mg/ml]) claforan and 300 mg/L (45µl of [200mg/ml]) Timentin for 1 hr with shaking to kill *Agrobacterium*. Blot dry and place on **CIM** containing claforan, timentin, and selecting agent (e.g., kanamycin, hygromycin etc), and incubate **in the dark**.

Day 20 Observe your cultures frequently. Subculture immediately if *Agrobacterium* re-growth is observed. Sacrifice those leaf disks with severe *Agrobacterium* attack.

Subculture the explants to fresh CIM media after 2 weeks. Callus usually starts to form on the wounded midribs **by the end of the first month**.

Day 34 Subculture the explants to **SIM** media with antibiotics to induce shoot regeneration **under light**. Subculture every 2-3 weeks. Adventitious shoots will appear within 4 to 6 weeks. Separate transformation events as soon as practical (calli or multiple shoot stages) during subculturing.

Adventitious shoot clumps with visible leaflets should be subcultured onto **SEM** media for shoot elongation. Do this according to the development of the adventitious shoots, and **DO NOT** let adventitious shoots over-proliferate on SIM (as I have seen in the last couple of years). This is because TDZ is very potent and prolonged cultivation in SIM will cause difficulty in shoot elongation with symptoms of vitrification and sicken shoots!!

Separate individual, elongated shoots and place into **RM** for root induction. Roots should appear in two weeks, but 1-2 subcultures (once per month) may be needed. This is the most sensitive step of transformant selection. False positive lines will not root readily, or if rooted, roots may grow on the surface of media with other abnormal morphology.

Transfer the plants into greenhouse within 1 month of root formation. **You should aim for at least 10 transformation events per construct, with controls!**

Micropropagation of *Populus tremula* x *P. alba* INRA 717-1B4

1. Cut stems into nodal segments of ~0.5 cm long, and place onto SIM media with TDZ for shoot induction.

2. Subculture multiple shoots to blank ½MS media for shoot elongation.

3. Excise individual shoots and place onto ½MS media for root induction.

4. Rooted plants can be maintained in the media for 1-2 months.

5. The top portion of the shoots with 4 leaves can be cut and placed onto ½MS media for root induction. Use the middle portion for micropropagation as in Step 1 above. The bottom portion with roots and 1-2 nodes can remain in the media for induction of new auxiliary shoots.