# TRANSFORMATION AND REGENERATION OF NICOTIANA

- Standard molecular laboratory equipment
- Biological air-flow cabinet (laminar flow hood)
- Shaking incubator (at 28 C)
- Plant growth cabinet
- Scalpels, forceps
- Head bead for sterilisation of scalpels and forceps
- You will also need Ethanol and Sodium Hypochlorite and Tween 20 for sterilisation of surfaces and leaf tissues.

**MS Broth**
1X Murashige and Skoog medium
3% Sucrose
pH 5.7

**Co-cultivation Medium**
1X Murashige and Skoog basal salt mixture
1X Gamborg's B5 vitamins (or substitute 1X Gamborg's B5 vitamins with standard MS medium with vitamins (Sigma M5519))
0.59g/L MES
3% Sucrose
pH 5.8

**Regeneration Medium**
1X Murashige and Skoog basal salt mixture
1X Gamborg's B5 vitamins (or substitute 1X Gamborg's B5 vitamins with standard MS medium with vitamins (Sigma M5519))
3% Sucrose
0.59g/L MES
1.0mg/L BAP
2.6 g/L phytagel
pH 5.8
*appropriate antibiotics – see table below*

**Rooting Medium**
Murashige and Skoog medium
3 % Sucrose
7 g/L Agar
pH 5.8
*appropriate antibiotics – see table below*

**Antibiotics:**

| Media | *Agrobacterium tumefaciens* strain | Antibiotics | | T-DNA marker | Selection antibiotic in Selection media herbicide |
|---|---|---|---|---|---|
| Selection Medium/ Rooting Medium | GV3101/ LBA4404 | Cefotaxime/ Augmentin at 500 mg/L | | *nptII* | Kanamycin at<br>100 mg/L (for N. benth)<br>100 mg/L (for N. tabacum) |
| | Agl1 | Timentin at 320 mg/L | | *bar* | Phosphinothricin at<br>2.0 mg/L (for N. benth.)<br>2.0 mg/L (for N. tabacum) |
| | | | | *hph* | Hygromycin at<br>10 mg/L (for N. benth)<br>30 mg/L (for N. tabacum) |

**Plant preparation (Option 1)**

1. Sterilize the seed washing it in 70% ethanol per 30 seconds.
2. Wash with water 3 times
3. Add 2% sodium hypochlorite and incubate in shaker at 100 rpm for 20 minutes
4. Wash with water 6 times to remove all residues
5. Spread the seed in a Magenta containing ½ MS media and grow in a 12 hour photoperiod for 60 days.

**Plant preparation (Option 2)**

1. Grow from seed in potting media in ~12cm pots in a growth rooms until plants are 10-20cm high. They should be no more than 3-4 weeks old and should not have started to flower. It is essential that the plants are not too mature because mature plants are resistant to Agrobacterium!
2. When ready to do the transformation, harvest *young* leaves up to 10 cm in diameter.
3. Surface-sterilise the leaves; briefly dip in 70% ethanol and then immerse in 1% of *freshly made up* sodium hypochlorite with a few drops of Tween 20 to act as a surfactant for 20-25 minutes. Stir **very** gently from time to time.
4. Wash the leaves well in several changes of sterile water. The leaves can be left in the flow cabinet for a couple of hours once they are sterilised.

**Agrobacterium preparation**

1. Three to four dayes before the transformation prepare an LB Plate with the appropriate antibiotics and grow the *Agrobacterium tumefaciens* for 48 hours at 28℃ to have individual colonies.
2. Two days before the transformation: Select two colonies and grow in 5 mL of LB liquid media containing the antibiotics for 24 hours in a 50 mL Falcon tube in the Shaker at 28℃ and 220 rpm.
3. One day before the transformation: In a new falcon add 5 mL of LB with the appropriate antibiotics and add 100 µL of the pre-Inoculum and grow overnight at 28℃ and 220 rpm.

4. On the day of transformation: when the culture has reached an $OD_{600}$ between 0.5 and 0.8 centrifuge 1 mL of Inoculum *Agrobacterium tumefaciens* at 5000 rpm and re-suspend it in 1 mL of NaCl 0,85%.

**Plant transformation**

1. On a sterile filter paper cut the leaves into 1-2cm squares with a sharp scalpel and immerse in a petri dish containing liquid MS. Ensure that all the leaves have been fully wetted.
2. Add 200 µL of Agrobacterium solution and mix it gently.
3. Close the plates and incubate *in the dark* for 48 hours at 28°C.
4. Wash the explants in MS liquid media and dry on a sheet of sterile filter paper.
5. Transfer the explants (10 per petri dish) to **Regeneration Medium** with appropriate antibiotics (e.g. kanamycin and cefotaxime).

**Week 3-6**

Subculture explants onto fresh Regeneration media every **7-10 days** for around **1-2 months** until the appearance of the first shoots.

Remove shoots with a sharp scalpel and plant into **Rooting Medium** with appropriate selection antibiotics.

Transgenic plants harboring antibiotic or herbicide resistance transgenes should start to root normally within 2 weeks and can be weaned out of tissue culture into sterile peat blocks before being transplanted to the glasshouse.