5. Forceps.
6. Laboratory sealing film (Whatman).

## 3. Methods

### 3.1. Establishment and Maintainance of Tobacco Shoot Cultures

#### 3.1.1. Sterilization of Tobacco Seed

1. Working in a sterile flow cabinet, place the seeds in a flat bottomed tube or beaker.
2. Immerse the seeds in 70% (v/v) ethanol for 20 s and agitate by gently swirling (*see* **Note 1**).
3. Tobacco seeds tend to float, so remove the ethanol using a sterile pipet by keeping the tip of the pipet close to the bottom of the beaker to suck off the ethanol, leaving the seeds at the bottom of the tube.
4. Immerse the seeds in the bleach solution for 20 min (*see* **Note 2**). Agitate occasionally by swirling.
5. Remove the bleach solution using a sterile pipet, as described in **step 3**, and wash the seeds five times in sterile distilled $H_2O$ (*see* **Note 3**). Leave a small volume of the final wash in the tube (0.1–0.3 mL).

#### 3.1.2. Germination of the Seeds

1. Using a sterile pipet, transfer the sterilized seeds in the final wash solution to a deep 9-cm Falcon Petri dish containing germination medium. Tilt the plate and remove the excess $H_2O$ with the pipet. Gently spread the seeds out with the pipet and seal the dish with Micropore tape.
2. Wrap the plates in aluminium foil, to exclude light, and leave at 4°C for 2–3 d to vernalize.
3. Remove the foil and transfer the plates to a growth cabinet set at 22–25°C and with a light intensity of 50–200 μmol/$m^2$/s, supplied by Warmwhite® or Coolwhite® (Osram, Merseyside, UK) fluorescent tubes. The light can be constant or long daylength. Germination will occur within 2–4 d.

#### 3.1.3. Initiation and Subculture of Shoot Cultures

1. When the seedlings are 2–3 wk old and large enough to handle, transfer them using sterile forceps, in a sterile flow cabinet, to a larger vessel, for example, a 60-mL sterile polypot containing MS30. In about 2–3 wk, the plantlets will have three to four pairs of leaves and can be subcultured.
2. To subculture the shoots, excise the apical bud and the newest pair of leaves from the plantet with a scalpel and embed the explant in MS30 in a Kilner jar or a similar vessel with a loose-fitting lid. A second and perhaps third cutting can be made from the same plantlet, if necessary; remove a stem section containing a pair of leaves and, hence, axillary shoot meristems, and place in another Kilner jar (*see* **Note 5**). Continue to grow the shoot cultures under the same conditions as described above. After 4–6 wk, the shoots will have produced roots and will have several pairs of fully expanded leaves. It is these fully expanded leaves that are used for transformation by *Agrobacterium*.