PRODUCTION OF VIRUS TESTED PLANTS

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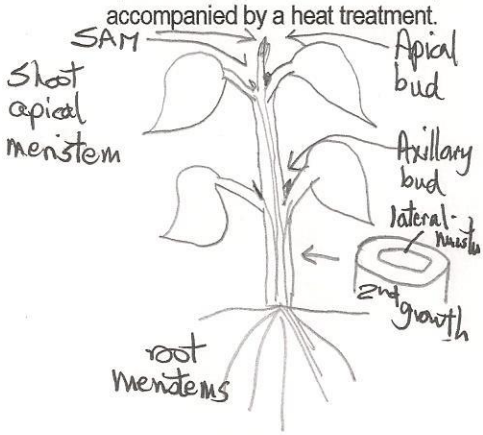
There are several methods available to produce virus-free plants, including heat treatment, meristem culture, heat treatment combined with meristem culture, adventitious regeneration in the presence of chemicals such as Virazole (1D Ribofuranosyl 1,2,4-triazole-3-carboxamide) or vibarabine and culture of cells or protoplasts from noninfected cells. Heat treatment inactivates isometric viruses. Agramine has been used successfully against yeast in tulips and iris.

Some headway has been made on use of antibiotics. One study used a combination of 2 mg cefotaxin, 25 mg tetracycline, 6 mg rifampicin and 6 mg polymixin B. Chemicals need to be filter sterilized and can also be used as a soak in experiment cleaning. Explants from mature, filed grown trees of *Theobroma cacoa* were vacuum infiltrated for one minute with liquid concoction containing the fungicide benlate (1g/l) and antibiotics carbenicilllin, rifampicin, tetracycline and cytotaxine (30, 30, 30 and 20 mg/l respectively) in MS medium. This was then 24 hours in the same solution. Contamination was reduced from 98% to 15% and survival increased from 3% to 84% (Matthews and Duncan 1993).

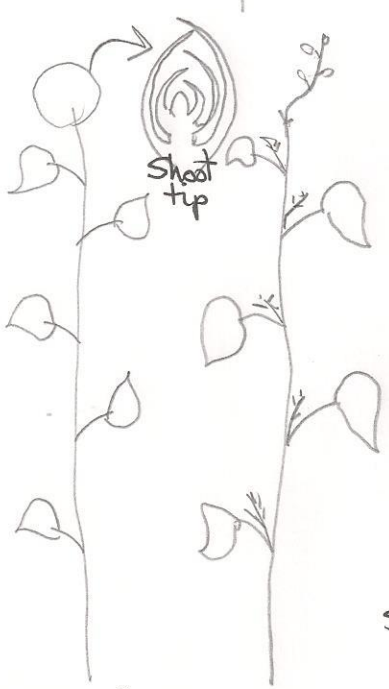
MERISTEM TIP CULTURE

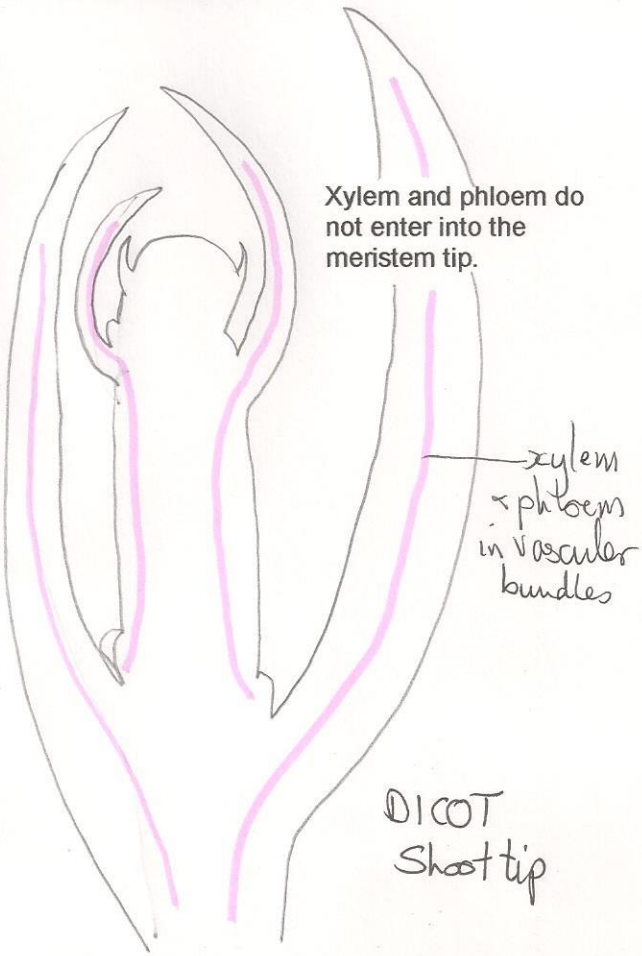
The main aim of meristem tip culture is to obtain virus free plants, but it has also enabled plants to be freed from other pathogens including viroids, mycoplasmas, bacteria and fungi. This technique is based on observations that the distribution of viruses in plants is uneven. In infected plants, the apical meristems are generally free or carry very low concentrations of viruses. In older tissues, the titer of viruses increases with increasing distance from the meristem tip. The main reason for this is thought to be the absence of a vascular system in meristem. Virus multiplication is slowed down or inactivated at high temperatures so that meristem culture is usually accompanied by a heat treatment.

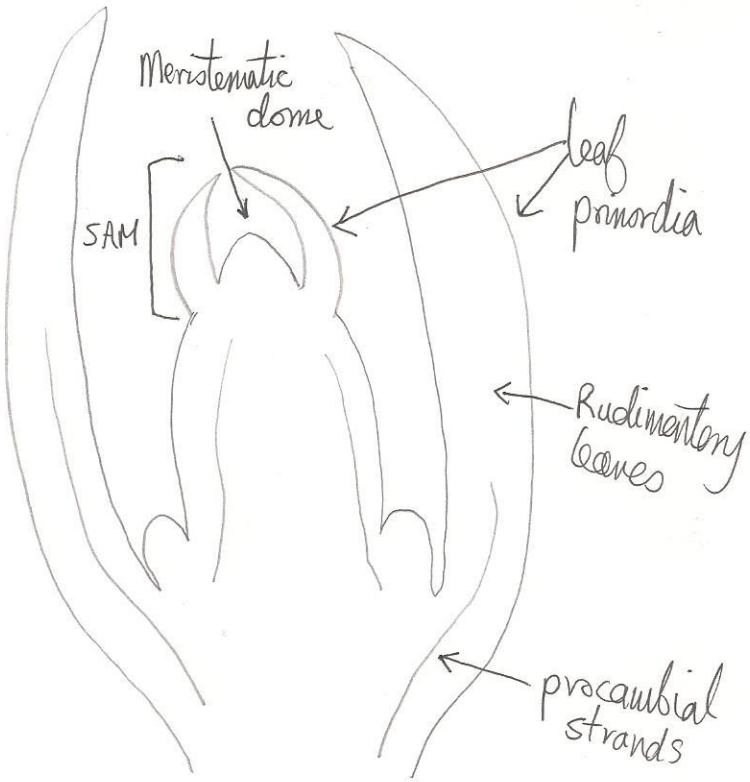


In angiosperms, the meristem dome in shoot tips is not autonomous for auxin and it is not the source of auxin for the plant. Auxin is probably synthesized by the second pair of youngest leaf primordial. An auxin and cytokinin are usually needed for successful meristem culture.

Meristems in plants produce new growth. There are both apical (located at the root and shoot tips) and lateral (at the nodes) buds. Removal of the apical bud breaks apical dominance and allows lateral buds to grow. The apical meristem lies distal to the youngest leaf primordium. It measures about 100 µm in diameter and 250 µm in length. In most published works virus-free plants are obtained by culturing 100-1000 µm long explants, these will include the meristematic dome and one or two leaf primordial. Speed is essential in order to prevent drying out of the meristem tips. Shoot tips are lightly sterilized to prevent carrying pathogens the meristem tip. Virus indexing of derived plants *in vitro* or after hardening is essential.







Protocol – Meristem tip culture

1. Materials needed for meristem tip culture in addition to those used for micropropagation are a stereomicroscope (8-40 x magnification), a light source, a set of needles and blades mounted on handles long enough to enter a test tube.

2. Select starting material from seedlings, new buds or young shoots. Apply heat treatment. Excise apical and lateral buds of about 1-5 cm long.

3. *Wash with soap, surface sterilize with 1-10% v/v sodium hypochlorite for 15 minutes.* Rinse in sterile distilled water three times. All procedures after the bleach soak are carried out in a laminar flow cabinet. The buds are lightly sterilized to prevent carrying pathogens to the meristem tip.

4. With the aid of a stereomicroscope, using sterile forceps and scapel, the outer leaves and primordia are removed until the glossy apical dome is visible. Leave enough subtending tissue to hold with a forcep. Speed is essential in order to prevent drying the meristem tips.

5. Excise the dome and one or two leaf primordia by making sharp, clean cuts on both side of the tip and across the base, this will prevent excessive browning. Transfer meristem tips to the culture medium on the end of the instrument such that the meristem tip is half immersed in medium. Slant the media as it cools so water does not settle around the meristem tip.

6. Seal the test tubes and place in the growth room. Monitor growth. Roll the meristem tip to a new location on the medium and transfer to fresh media depending on growth.

7. Virus indexing of derived plants is essential to determine if they are free of the virus in question. It is best to test the plants after hardening and growingon

Notes

- To eliminate viruses, the size of the dome should not exceed 0.20.5 mm in length. Some excised meristems (e.g. *Cattleya*) produce toxic substances, a liquid medium should be used in these cases and the meristem tip be placed on paper bridges. The percentage of isolated meristems developing into virus free plants is generally small.
- The combination of heat treatment and meristem culture is more effective if the virus is heat sensitive. Heat treatment varies: plants can be exposed to 32350C for 23 days, 33370C for four weeks or 37380C for 2040 days depending on plant and virus. The temperature and treatment time should be chosen to allow plant to just survive while inactivating the virus.

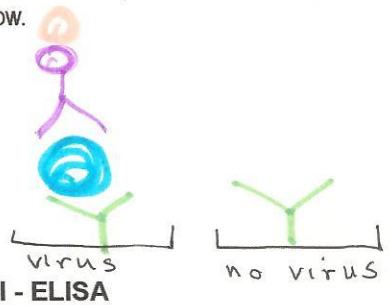
- For more pics, view https://www.pinterest.com/mitchell2758/plant-morphology

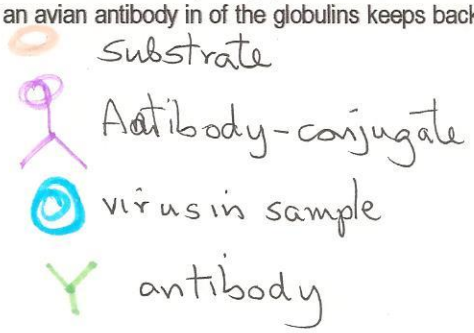
METHODS OF VIRUS TESTING

The simplest methods involve the use of indicator plants which react promptly and characteristically to sap inoculation, usually with the formation of local lesions on the inoculated leaves. The best 'general purpose' indicator plants are found in the Chenopodiaceae, particularly *Gomphrena globosa*.

ELISA

Enzymelinked Immunosorbent assay (ELISA) is a sensitive test which is widely used. Most workers use the direct doubleantibody sandwich method which requires the preparation of a different antibody conjugate for each virus tested. Multilayered sandwich procedures are often more sensitive. The use of an avian antibody in of the globulins keeps background readings low.





Protocol - ELISA

1. Put 200 µl of antibody (globulin) of stock 1 µg /ml in coating buffer into each cell of a 96 well microtitre plate. Incubate in plate covered with cling film in a box with damp tissue for 2 hour at 37◦C.
2. Wash the plate with phosphate buffered saline (PBS) plus Tween by flooding plate (avoid air bubbles) and leave it for 3 minutes. Flick plate to remove PBS-Tween. Repeat the procedure three times.
3. Store sample maximum three days in freezer if also testing for viroid. Prepare samples by grinding in extraction buffer. Can put in plastic bag and crush with a test tube. Add 200 µl of sample in duplicate in a plate. Incubate overnight at 4◦C in container with damp tissue.
4. Wash plate three times with PBS-Tween as in (2).
5. Add 200 µl antibodyconjugate (enzyme labelled globulin) from stock 1 µg/ml. incubate at 37◦C for 36 hours.
6. Wash plates three times as above.
7. Add 200 µl freshly prepared substrate to each well. Incubate at room temperature (20-24 ◦C) for 1 hour.
8. Stop the reaction with 50 µl of 3 M solution of NaOH. Stop the reaction before wells that are yellow go above reading capacity of spectroscope. Best if you can use ELISA reading.
9. Assess results (a) visual observation of colour reaction.
   (b) measure absorbance at 405 nm.

Solutions

Coating buffer: in 1 litre, pH 9.6
NaHCO3 2.93 g
Na2CO3 1.59 g

Phosphate buffered saline (PBS): in 1 litre, pH 7.0
Na2HPO4 . 2H2O (0.01 M) 8.9 g
NaH2PO4 . 2H2O (0.01 M) 7.8 g
NaCl 42.5 g

Extraction buffer: in 100 mls.: Crush powder and slowly add PBS-Tween, 0.2 % ovalbumin (used to bind phenols), 2 % w/v polyvinylpyrrolidone (PVP)

Washing buffer: PBS-Tween (= PBS + 0.5 mg /l Tween)

Conjugate buffer: Same as extraction buffer

Substrate buffer: 0.1 M diethanolamine, pH 9.8. Store in dark glass bottle at 4◦C.

Substrate: 0.6 mg/ml 4nitrophenyl in substrate buffer

Siliconise all glassware used for antiserum.

ELECTRON MICROSCOPY (EM)

Virus particles in crude sap can be directly observed with EM eg. quick dip method of Brandles. Sample mounted on 3 mm copper grid covered with thin film of plastic (Formvar). Grinds contain apertures (60-160 meshes/cm). Virus particles stick to Formvar film. The viruses are highly transparent to electrons so shadowcasting or negative staining is essential. Shadowcasting can be done with dried preparations and then sent to suitable lab for examination.