

## **Meristem-Tip Culture for Propagation and Virus Elimination**

**Brian W. W. Grout**

### **1. Introduction**

The essence of meristem-tip culture is the excision of the organized apex of the shoot from a selected donor plant for subsequent *in vitro* culture. The conditions of culture are regulated to allow only for organized outgrowth of the apex directly into a shoot, without the intervention of any adventitious organs (1–3). The excised meristem tip is typically small (often <1 mm in length) and is removed by sterile dissection under the microscope, as in the potato example detailed in this chapter (Fig. 1). The explant comprises the apical dome and a limited number of the youngest leaf primordia, and excludes any differentiated provascular or vascular tissues. A major advantage of working with such a small explant is the potential that this holds for excluding pathogenic organisms that may have been present in the donor plants from the *in vitro* culture (*see below*). A second advantage is the genetic stability inherent in the technique, since plantlet production is from an already differentiated apical meristem and propagation from adventitious meristems can be avoided (3–9). Shoot development directly from the meristem avoids callus tissue formation and adventitious organogenesis, ensuring that genetic instability and somaclonal variation are minimized. If there is no requirement for virus elimination, then the less demanding, related technique of shoot-tip culture may be more expedient for plant propagation. In this related procedure the explant is still a dissected shoot apex, but a much larger one that is easier to remove and contains a relatively large number of developing leaf primordia. Typically, this explant is between 3 and 20 mm in length, and development *in vitro* can still be regulated to allow for direct outgrowth of the organized apex.

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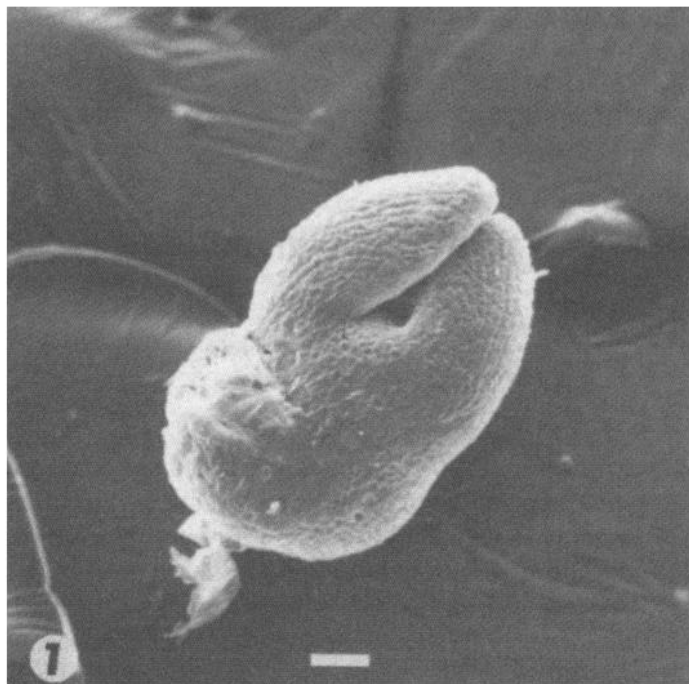


Fig. 1. A freshly excised meristem tip from an axillary bud of the potato *Solanum tuberosum*. The two smallest emergent leaf primordia are present. Scale bar represents 50  $\mu$ M.

The axillary buds of in vitro plantlets derived from meristem-tip culture may also be used as a secondary propagule. When the in vitro plantlet has developed expanded internodes, it may be divided into segments, each containing a small leaf and an even smaller axillary bud (Fig. 2). When these nodal explants are placed on fresh culture medium, the axillary bud will grow directly into a new plantlet, at which time the process can be repeated. This technique adds a high propagation rate to the original meristem-tip culture technique, and together the techniques form the basis of micropropagation, which is so important to the horticulture industry (1-4; see Table 1).

It is possible, however, that callus tissue may develop on certain portions of the growing explant, particularly at the surface damaged by excision (Fig. 3). The only acceptable situation under such circumstances is where the callus is slow-growing and localized, and the callus mass and any organized development on it can be excised at the first available opportunity. Studies from the author's laboratory on cauliflower plants regenerated from wound callus and floral meristems in vitro showed normal phenotypes for all the plants produced by

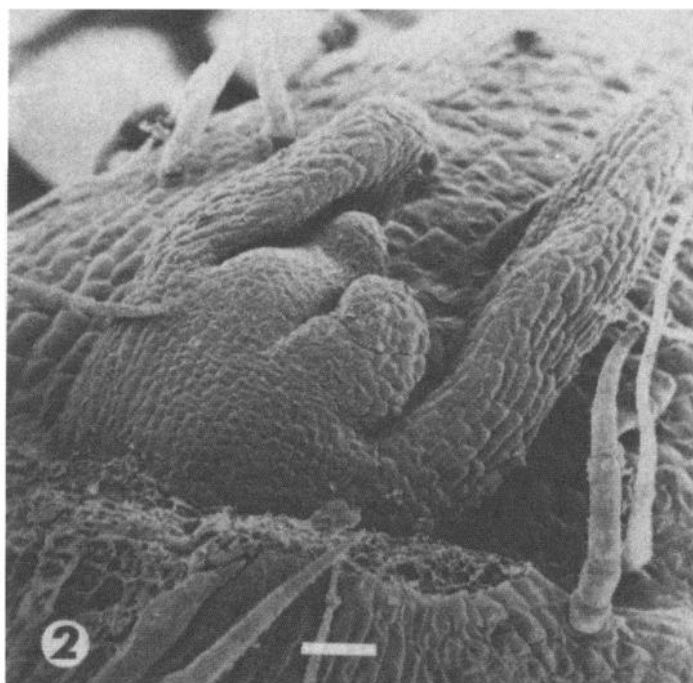


Fig. 2. An axillary bud from a freshly excised nodal segment, taken from a meristem-derived potato plantlet in vitro. A number of small leaf primordia are apparent, emphasizing the effectively normal structure of this very small axillary organ. Scale bar represents 50  $\mu$ M.

**Table 1**  
**The Propagation Potential Inherent**  
**in the Meristem-Tip Culture Technique<sup>a</sup>**

Time from culture initiation, mo	No of nodes available <sup>b</sup> for further subculture, equivalent to plantlet number
3	5
4	25
5	125
6	625
7	3125
8	15,625

<sup>a</sup>The data are for potato species, *Solanum curtilobum*, and were calculated from propagation rates achieved in the author's laboratory.

<sup>b</sup>Assumes 5 nodes available/plantlet.

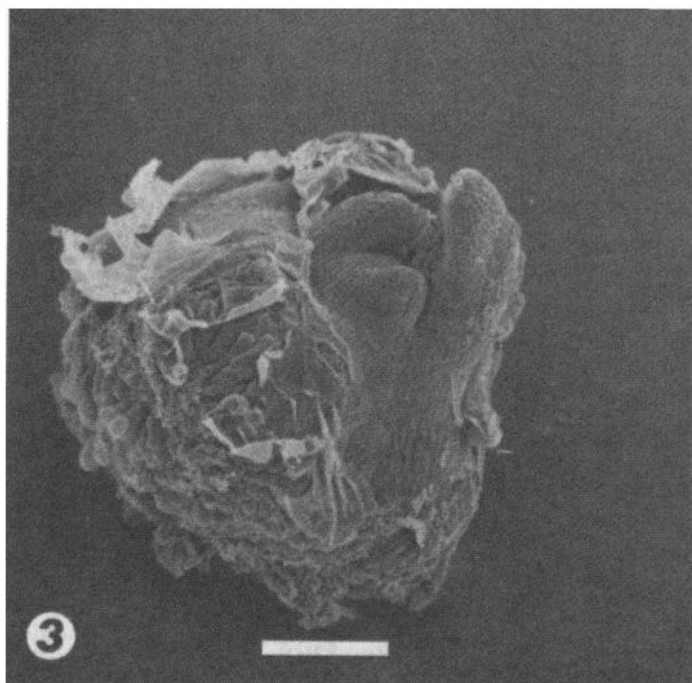


Fig. 3. A meristem-tip culture of potato 6 d after initiation. A distinct swelling can be seen at the wound surface, and larger callus cells are beginning to proliferate from it. Scale bar represents 500  $\mu$ M.

meristem culture, whereas those of callus origin included rosette and stunted forms, and both glossy and serrated leaves as examples of phenotypic deviance.

These abnormal plants, derived from callus tissue, and normal plants from meristem culture showed the same DNA levels, as measured by scanning microdensitometry, but showed considerable differences when the isozymes of acid phosphatase was examined by gel electrophoresis. This suggests a range of unacceptable variation at the gene level, introduced by the adventitious origin of the shoots.

A further advantage of meristem culture is that the technique preserves the precise arrangement of cell layers necessary if a chimeral genetic structure is to be maintained. In a typical chimera, the surface layers of the developing meristem are of differing genetic background, and it is their contribution in a particular arrangement to the plant organs that produces the desired characteristics, e.g., the flower color in some African violets. As long as the integrity of the meristem remains intact and development is normal *in vitro*, then the chimeral pattern will be preserved. If, however, callus tissues are allowed to

**Table 2**  
**The Relationship of Explant Size**  
**to Effective Virus (WCMV) Elimination in Clover<sup>a</sup>**

Explant size, mm	No. explants taken into culture	No. of plants established in soil	No. virus-free plants
<0.6	90	18	18
0.6–1.2	113	45	19
1.3–1.8	190	102	25
1.9–2.4	158	88	11
2.5–3.0	174	92	11

<sup>a</sup>Data from (15)

form and shoot proliferation, subsequently, is from adventitious origins, then there will be a risk that the chimeral layers of the original explant may not all be represented in the specially required form in the adventitious shoots.

The technique of meristem culture may be exploited in situations where the donor plant is infected with viral, bacterial, or fungal pathogens, whether or not symptoms of the infection are expressed. The basis of eradication is that the terminal region of the shoot meristem, above the zone of vascular differentiation, is unlikely to contain pathogenic particles. If a sufficiently small explant can be taken from an infected donor and raised *in vitro*, then there is a real possibility of the derived culture being pathogen-free. Such cultures, once screened and certified, can form the basis of a guaranteed disease-free stock for further propagation (9–11). The meristem-tip technique can be linked with heat therapy to improve the efficacy of disease elimination, or antiviral, chemotherapeutic agents may be investigated (9–14).

Whatever variants of technique are employed for virus eradication, the key to success is undoubtedly the size of the explant. The smallest explants are those that typically, will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material when entire plants are reared in the glasshouse or field. This is clearly illustrated by attempts at the elimination of WCMV from *Trifolium pratense* (Table 2; 15).

If meristem culture alone is not successful in producing any virus-free plants, then temperature-stress treatment of donor plants and/or the use of antiviral agents will have to be considered. There are no prescriptive, global methodologies for these treatments, and an empirical study will be required. Heat therapy relies on the growth of the donor plants at elevated temperatures, typically 30–37°C and may involve a treatment of several weeks. Where *in vitro* cultures provide the donor material, it is possible to heat treat the cultures also. A number of factors may contribute to the absence of virus particles from the

**Table 3**  
**The Effect of Extended Low-Temperature**  
**(2–4°C) Treatment on the Elimination**  
**of Hop Latent Viroid from Hop Plants<sup>a</sup>**

	Duration of cold treatment, mo	Propagation of viroid-free plants
Variety 1	6	0/4
	17	4/23
Variety 2	21	1/14
	9	3/12

<sup>a</sup>Adapted from (14).

meristems of treated plants, including reduced movement of the particles to the apical regions, a thermally induced block on viral RNA synthesis, and inactivation of virus particles. It is useful to note that extended, low-temperature treatment of donor material may also be effective (14). In attempts to eliminate hop latent viroid, the low-temperature (2–4°C) treatment of parent plants was only effective if extended beyond 6 mo (Table 3). Particularly when subjecting donor plants to temperature stresses, it is extremely important to understand the physiology and developmental behavior of the plants concerned, so that maximum stress can be applied while maintaining an acceptable pattern of growth. A good knowledge of the taxonomy of the plant under investigation is also valuable when selecting likely treatments from the literature, since close, or distant, relatives can be identified.

Antiviral chemicals can be used as additives in the culture medium (11,12), and one of the most widely used is ribavirin, also known as virazole. This compound is a guanosine analog with broad-spectrum activity against animal viruses and appears also to be active against plant virus replication in whole plants. Increasing concentrations of ribavirin and increasing length of culture incubation in the presence of the compound typically increase the effectiveness of virus elimination, but slowed growth and phytotoxicity may be evident at high concentrations.

The major advantages of meristem culture are that it provides:

- 1 clonal propagation in vitro with maximal genetic stability;
- 2 the potential for removal of viral, bacterial, and fungal pathogens from donor plants;
3. the meristem tip as a practical propagule for cryopreservation and other techniques of culture storage,
4. a technique for accurate micropropagation of chimeric material; and
5. cultures that are often acceptable for international transport with respect to quarantine regulations.

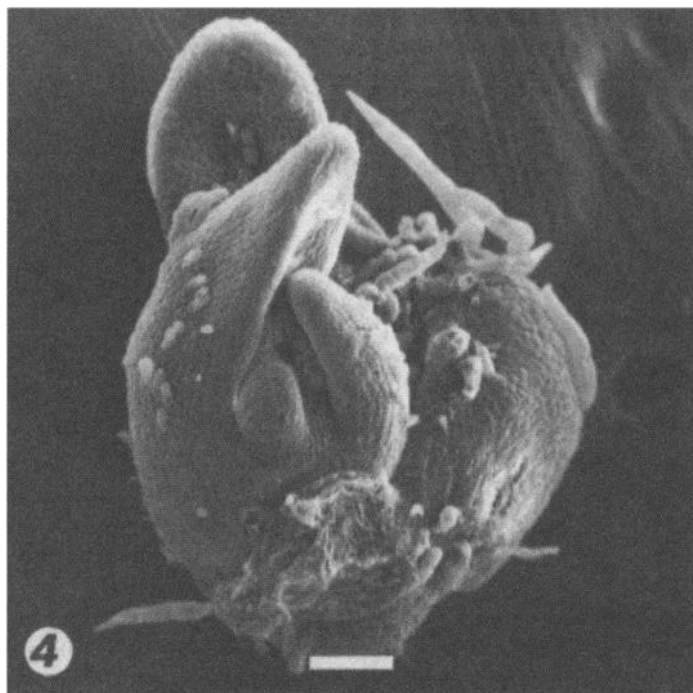


Fig. 4. A meristem-tip culture of potato 10 d after initiation. When excised, this explant had only two obvious leaf primordia, and has both developed and extended considerably during the culture period. Scale bar represents 500  $\mu$ M.

The technique of meristem culture, with optional procedures for virus elimination, is detailed below for potato species (*see Fig. 4*). The steps described are broadly applicable to a wide range of plant subjects, although formulations of media and reagents may need to be altered.

## 2. Materials

1. Glasshouse-grown potato plants provide the stem sections with axillary buds to be dissected. These are raised in an insect-proof facility where possible, and maintained on capillary matting on raised benches so that overhead watering is avoided. The donor plants can be subjected to heat treatments for virus eradication, involving growth at 33°C for 4–6 wk.
2. Wide-necked test tubes make suitable culture vessels for incubating meristem cultures, capped with purpose-bought tops, or aluminum foil, and sealed after inoculation with a Parafilm strip.
3. The culture vessels contain 2.5 mL of Murashige and Skoog medium (*16* and *see Appendix*) solidified with 0.8–1.0% w/v agar and supplemented with 0.2 mg/L

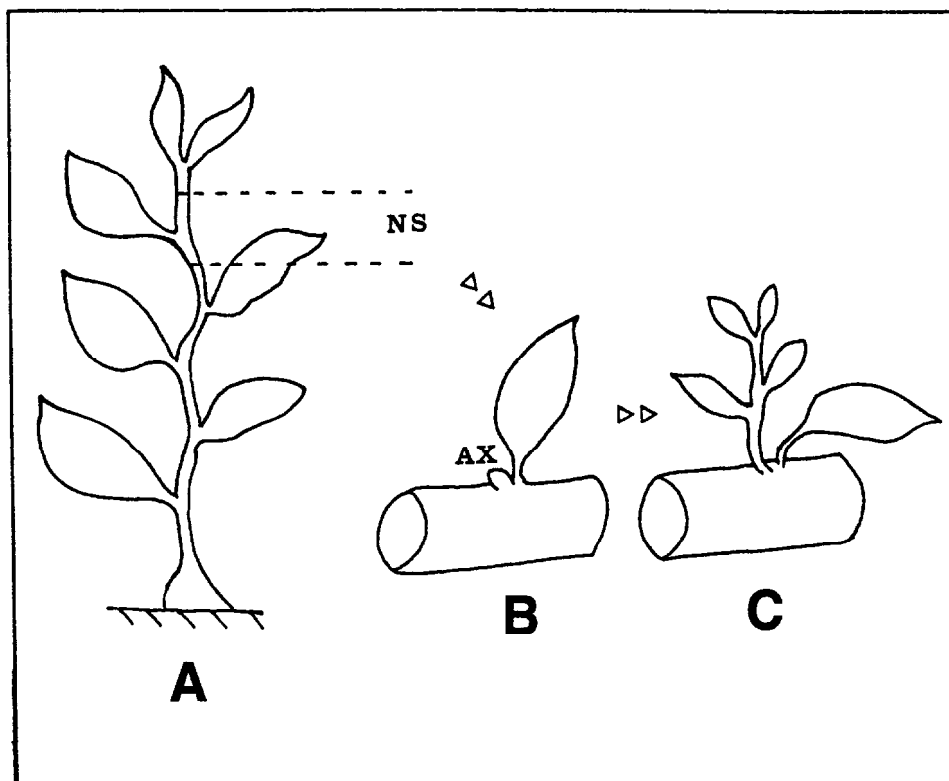


Fig. 5. Propagation from meristem-tip derived plantlets by the technique of nodal culture (A) A plantlet showing extension growth in vitro. The nodal segment to be excised is indicated (NS). (B) The excised nodal segment as it is transferred onto fresh culture medium, showing the axillary bud (AX) that will be responsible for subsequent growth. (C) The pattern of development of a successful nodal segment culture, showing extension growth of the new plantlet

1-naphthalene acetic acid and 0.5 mg/L gibberellic acid, pH 5.8 (see also 17–20). Alternatively, liquid media may be used in conjunction with paper bridges or fiber supports in the culture vessel. If required for virus elimination, ribovarin at 200  $\mu$ M is filter-sterilized and added to the medium before solidification.

4. Nodal cultures, derived from in vitro plantlets, are excised as detailed in Fig. 5 and cultured in identical vessels to the excised meristems. The growth medium is the same as that for meristem culture without growth hormone supplements.
5. Presterilization of the excised stem segments is carried out using an absolute ethanol dip for 30 s with a subsequent sterilization using 5% v/v sodium hypochlorite with a drop of Tween 80 (added as a wetting agent). This is particularly important when the tissue surfaces are waxy or coated with epidermal hairs. Sterile distilled water for rinsing is also required.

4. A dissection microscope with a magnification of at least 15 $\times$  and mounted in a laminar flow cabinet is required. A piece of expanded polystyrene covered in white plastic film and taped to the microscope stage is ideal for holding sterilized stem segments in place for dissection, using sterilized pins. The tips of 12-gage hypodermic needles are used to carry out the dissection.
5. A growth room is needed that provides a controlled environment in which to incubate the cultures. Lighting at 4000 lx at culture level with a 16-h photoperiod, provided by warm white fluorescent tubes, and a constant temperature of  $25 \pm 1^\circ\text{C}$  provides optimal culture conditions.

### 3. Methods

1. Select a suitable donor plant, in this case, any of the *Solanum tuberosum* ssp *tuberosum* types (see **Note 1**), following any desired temperature pretreatments. Excise stem segments containing at least one node from the donor plant.
2. Remove mature and expanding foliage to expose the terminal and axillary buds. Cut donor segments to 4-cm lengths, and presterilize by immersion in absolute ethanol for 30 s.
3. Sterilize by immersing the donor tissues in the sodium hypochlorite solution, with added detergent, for 8 min (see **Note 2**).
4. Following surface sterilization, rinse the tissues three times in sterile distilled water.
5. Mount the stem segment on the stage of the dissection microscope, and use the tips of hypodermic needles to dissect away progressively smaller, developing leaves to expose the apical meristem of the bud, with the few youngest of the leaf primordia (see **Note 3**).
6. Excise the explant tissue that should comprise the apical dome and the required number of the youngest leaf primordia (see **Note 4**).
7. After excision, the explant is transferred directly onto the selected growth medium, and the culture vessel is closed.
8. Transfer the completed meristem-tip culture to the growth room.
9. If the explant is viable, then enlargement, development of chlorophyll, and some elongation will be visible within 7–14 d (**Fig. 4**; see **Note 5**).
10. Maintain the developing plantlet in vitro until the internodes are sufficiently elongated to allow dissection into nodal explants.
11. To prepare nodal explants, remove the plantlets from the culture vessels under sterile conditions, and separate into nodal segments (**Fig. 5**). Each of these is transferred directly onto fresh growth medium to allow axillary bud outgrowth. Extension of this bud should be evident within 7–14 d of culture initiation (see **Note 6**).

### 4. Notes

1. Donor plants should be selected for general health and vigor. Donor tissues should be taken from young, actively growing stems to ensure the best chance of success. However, if donor plants have been subjected to temperature stresses as a pretreatment, then allowances must be made for apparent foliar injury and slowed

growth Using donor plants that have been glasshouse-raised and only watered from below will help minimize infection problems

2. Be careful. It is often easy to oversterilize during surface sterilization and lethally damage tissues and, therefore, the meristems in an attempt to eradicate surface pathogens
- 3 It is only with practice that reproducible, high levels of culture success will be achieved, since early attempts at dissection will often result in damaged tissue. Hypodermic needles used as dissecting tools often need to be discarded after two to three meristem excisions
- 4 It seems that the presence of leaf primordia is essential for successful culture growth, so removal of too small an explant may restrict success
- 5 A consequence of dissection may be the production in the tissue of toxic, oxidized polyphenolic compounds (3) These are more prevalent in cultures of woody species. Their effects may be minimized by rapid serial transfer of the excised meristem tip to fresh medium as soon as significant browning of the medium occurs, or by reducing light available to the culture in the early stages of development Attempts can be made to reduce oxidation of the secreted polyphenolic compounds by incorporating antioxidants into the growth medium These might include ascorbic acid, dithiothreitol, and polyvinyl pyrrolidone
6. Media are commonly species-specific with regard to the formulation of the required growth regulators and their concentrations, and may also be specific for the other organic and inorganic constituents. The specificity may even extend to the cultivar/race level with respect to optimal growth. For potato, a range of successful media for different varieties and species, at a range of ploidy levels, have been published (17–20) Reference sources can provide an appropriate growth medium to begin an empirical study, using the formulation for the closest taxonomic relative if no medium is detailed for the particular plant under investigation

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# APPENDIX

## Widely Used Plant Cell Culture Media

The following represent the most widely used media for plant cell culture. Recipes for additional media used for specific applications are to be found in the relevant chapters and are listed in the index.

	Murashige and Skoog complete medium (1)	Murashige and Skoog plant salt mixture (1)	Linsmaier and Skoog medium (2)
<b>Macroelements (mg/L)</b>			
CaCl <sub>2</sub>	332.020 <sup>a</sup>	332.020 <sup>a</sup>	332.020 <sup>a</sup>
KH <sub>2</sub> PO <sub>4</sub>	170.000	170.000	170.000
KNO <sub>3</sub>	1900.000	1900.000	1900.000
MgSO <sub>4</sub>	180.540 <sup>b</sup>	180.540 <sup>b</sup>	180.540 <sup>b</sup>
NH <sub>4</sub> NO <sub>3</sub>	1650.000	1650.000	1650.000
<b>Microelements (mg/L)</b>			
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.025	0.025
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.025	0.025
FeNaEDTA <sup>c</sup>	36.700	36.700	36.700
H <sub>3</sub> BO <sub>3</sub>	6.200	6.200	6.200
KI	0.830	0.830	0.830
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.900	16.900	16.900
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.250	0.250	0.250
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.600	8.600	8.600
<b>Organics (mg/L)</b>			
Glycine	2.000		
Myo-inositol	100.000		100.000
Nicotinic acid	0.500		
Pyridoxine-HCl	0.500		
Thiamine-HCl	0.100		0.400

<sup>a</sup>440 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O

<sup>b</sup>370 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O

<sup>c</sup>Original recipe uses 5 mL/L of a stock: 5.57 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 7.45 g Na<sub>2</sub>EDTA dissolved in 1 L

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	Gamborg's B5 medium (3)	Schenk and Hildebrandt medium (4)	Nitsch's medium (5)
Macroelements (mg/L)			
CaCl <sub>2</sub>	113.230 <sup>a</sup>	151 000 <sup>b</sup>	166 000 <sup>c</sup>
KH <sub>2</sub> PO <sub>4</sub>			68 000
KNO <sub>3</sub>	2500.000	2500 000	950 000
MgSO <sub>4</sub>	121.560 <sup>d</sup>	195.050 <sup>e</sup>	90.27 <sup>f</sup>
NH <sub>4</sub> NO <sub>3</sub>			720.000
NaH <sub>2</sub> PO <sub>4</sub>	130.440		
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	134 000		
(NH <sub>4</sub> )H <sub>2</sub> PO <sub>4</sub>		300 000	
Microelements (mg/L)			
CoCl <sub>2</sub> 6H <sub>2</sub> O	0 025	0 010	
CuSO <sub>4</sub> 5H <sub>2</sub> O	0.025	0 020	0 025
FeNaEDTA	36 700	19 800	36 700
H <sub>3</sub> BO <sub>3</sub>	3 000	5 000	10 000
KI	0.750	1.000	
MnSO <sub>4</sub> H <sub>2</sub> O	10 000	10 000	18 940
Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.250	0.100	0.250
ZnSO <sub>4</sub> 7H <sub>2</sub> O	2 000	1 000	10 000
Organics (mg/L)			
Biotin			0 050
Folic acid			0.500
Glycine			2.000
Myo-inositol	100.000	1000 000	100.000
Nicotinic acid	1.000	5.000	5 000
Pyridoxine-HCl	1.000	0 500	0 500
Thiamine-HCl	10.000	5.000	0.500

<sup>a</sup>150 mg/L CaCl<sub>2</sub> 2H<sub>2</sub>O

<sup>b</sup>200 mg/L CaCl<sub>2</sub> 2H<sub>2</sub>O.

<sup>c</sup>220 mg/L CaCl<sub>2</sub> 2H<sub>2</sub>O

<sup>d</sup>250 mg/L MgSO<sub>4</sub> 7H<sub>2</sub>O

<sup>e</sup>400 mg/L MgSO<sub>4</sub> 7H<sub>2</sub>O

<sup>f</sup>185 mg/L MgSO<sub>4</sub> 7H<sub>2</sub>O.

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