Meristem culture for virus elimination

Introduction

Meristem culture technology has been applied to many crops, especially vegetatively propagated crops such as potato, over the last 40 years to eliminate viruses from important cultivars (Slack 1980; Bhojwani and Razdan 1983; Hartmann et al. 1990). Tissue culture technology has been utilized for both basic and applied purposes in potato programs (Dodds 1988). Because potatoes may be infected by over 20 viruses, 6 worldwide, meristem culture and tissue culture techniques have been adopted for virus elimination and for maintenance and propagation of nuclear seed stocks, respectively (Bryan 1988; Jones 1988). Meristem culture and heat therapy have been integral to a virus-free potato cultivar collection in Canada since 1967 (Wright 1988). The protocols described in the following exercises are utilized routinely in our laboratory to establish and maintain a pathogen-free potato collection that serves as the primary resource for the production of certified seed potato stocks in New York State (USA). These protocols should be applicable to other species by varying the culture media as required, and by adapting these techniques to the growth habit and meristem location(s) in the species studied. Plantlets in tissue culture provide the base for rapid multiplication of potato seedstocks (Bryan 1988), and for chemical and heat therapy procedures that improve the efficiency of virus elimination (Griffiths et al. 1990; Sanchez et al. 1991). Excision of the meristem and regeneration of plantlets on hormone amended media have permitted the propagation of plants free from systemically invasive viruses and from other disease-causing pathogens. The meristem, which is about 0.1 mm in diameter and about 0.25 mm long, is composed of actively dividing and undifferentiated cells at the apices of shoots and roots. This tissue has no vascular system and is, therefore, less likely to be infected with systemic viruses. The protocols described take advantage of the sterile conditions in which in vitro plants grow, the minimal space required to maintain and treat plantlets, the juvenile growth pattern of plantlets, and the ability to independently manipulate single variables.

Objectives and Goals

- To establish potato plantlets in vitro from plants or sprouted tubers.
- To excise meristems from in vitro plantlets and to regenerate plantlets from meristems.
- To determine virus elimination efficiency from plantlets following meristem culture and nodal cutting propagation.
- To determine effect of heat therapy on virus elimination from plantlets following meristem culture and nodal cutting propagation.

Equipment and Reagents

- Laminar air flow hood, preferably, or clean contained work area
- Two environmental growth incubators, one for propagation of plantlets and one for heat therapy treatment, or illuminated shelves for propagation and a chamber or other unit for heat therapy
- Media dispenser, recommended but not required

- Stereo microscope with illuminator, for meristem excision
- Glass culture tubes, 20 X 100mm, and plastic closures or caps
- Disposable petri plates, 15 X 60 mm, for meristem excision
- Alcohol lamp, gas or electric bunsen burner, or other sterilizing unit
- Dissecting tools
- Small fine
- tipped forceps, 1l0mm
- Large Gruenwald bayonet forceps, 216mm
- Scalpel handle #7 and scalpel blades #11
- Dissecting scissors, 110 mm
- Sterile filter papers, 9 cm, or sterile petri plates to use as a work surface
- Difco Bacto
- Agar
- Gelrite or Phytagel (Sigma #P8169)
- Murashige and Skoog Basal Salt Mixture (Sigma #M5524)
- Potassium hydroxide, 1 N - Ethanol, 70%
- Sodium hypochlorite, 0.5% solution

Procedures

Preparation of Media Meristem

Medium Vitamin Stock

- Glycine 40.0mg
- Pyridoxine HCL 10.0mg
- Nicotinic Acid 10.0 mg
- Thiamine HCl 8.0mg
- Bring to 100 ml with distilled water, divide into aliquots and freeze

MS Medium[a]	***Meristem Medium***[b]
MS Major Salts	MS Major Salts
MS Minor Salts	MS Minor Salts
Iron EDTA	Iron EDTA
NaH2P04. H20, 0.17 g/l	Meristem Medium Vitamin Stock, 5.0 ml/l
Thiamine. HCl, 0.4 mg/l	Inositol, 0.1 g/l
Inositol, 0.1 g/l	Sucrose, 3%
Sucrose, 3%	KIN, 0.01 mg/l
Gelrite, 0.25%	GA_3, 0.1 mg/l
	Difco Bacto Agar, 7.0 g/l

Notes. For MS and growth regulator stock solutions see Chapter 2. MS Salts from Sigma (#M5524) is satisfactory. Adjust pH of media to 6.0 with 1 N KOH. Dispense 10 ml into 20 X

100mm culture tubes, cap or plug with cotton. After autoclaving, leave tubes at room temperature to cool at a 45 ° slant. The medium may be stored 6-8 weeks, preferably at 4°C. In a laminar flow hood, dispense 8- 10 ml into sterile disposable petri plates (IS X 60mm). This medium should be stored at 4°C and used within 10-14 days.

Note. For specialized media other than for normal propagation purposes, one of the following may be added as needed to the medium:

- For increased rooting add IAA at 1.0 mg/l
- For virus eradication add Ribavirin at 20.0 mg/l
- For long-term storage add Mannitol at 40.0 gil.

Treatment of Materials

For the general propagation of plantlets, an environmental growth incubator programmed to provide 16 h light/8 h dark per day with a light intensity of 75 μmol $m^{-2}s^{-1}$ and a temperature of22-25 °C is ideal. However, plantlets can be grown on an illuminated shelf at most room temperatures in a clean area. For the regeneration of meristems to plantlets, similar conditions are suitable but a gentle heat source from below, such as that from a ballast on a light unit, can enhance rooting and regeneration.

Protocols

Initiating Cultures from Tubers

1. Potato seed tubers may be stored at 4°C for about 1 year. Most cultivars are dormant after harvest and must be stored for about 2 months before they will grow. Warm potato tubers to room temperature for 2 days before using to avoid rotting.
2. Surface sterilize tubers by soaking in 0.5% sodium hypochlorite for 20 min after rinsing in tap water. Allow tubers to air dry (Fig. 1).
3. Allow tubers to sprout under low light intensity, e.g., fluorescent lights, or plant as 30-60g seed pieces (whole or cut tubers) into sterile soil mix.
4. Allow sprouts to become 2-5 cm long and plants to reach six to eight-leaf stage.
5. Plants or sprouts may be tested for potato viruses by enzyme-linked immunosorbent assay (ELISA - see protocol below) or other suitable assay.
6. Plantlets may be maintained by making nodal cuttings (Fig. 2) and transferring them to new culture tubes (see protocols below). New plantlets should be available every 4-6 weeks. Plantlets may be stored for about 1 year using MS medium +40g/1 mannitol, sealing the tube with Parafilm, and holding plantlets at 6-10°C with 12h photoperiod.

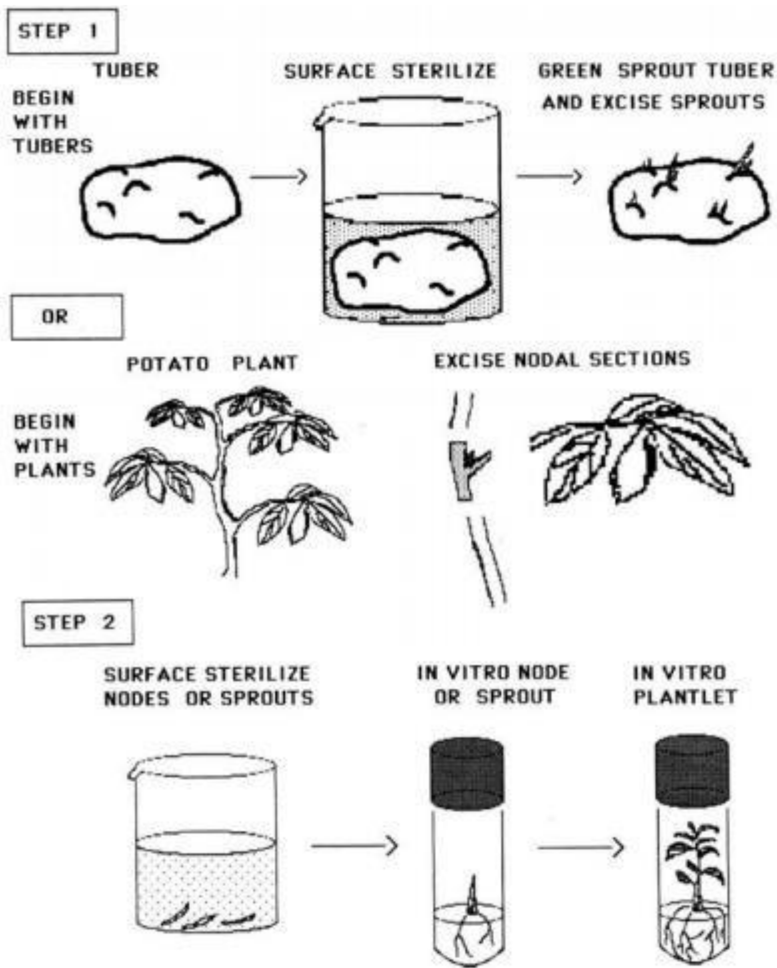

Fig. 1. Introduction of plant materials into sterile tissue culture

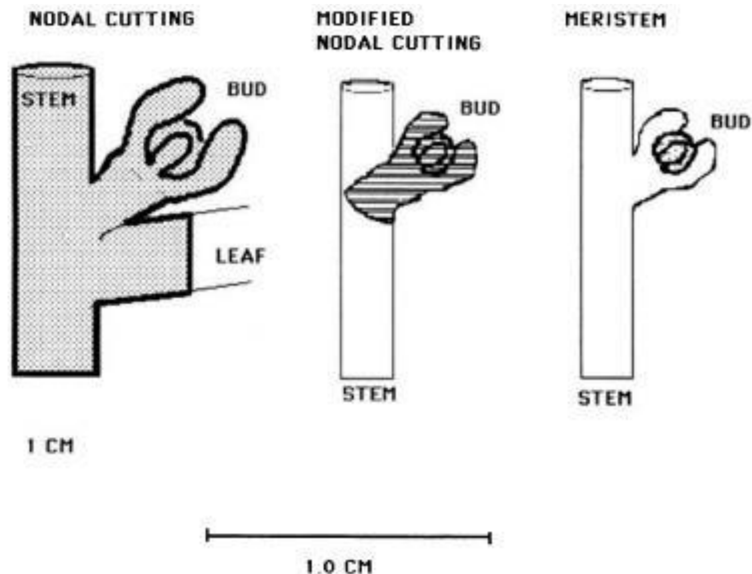

Fig. 2. Nodal cutting, modified nodal cutting, and meristem used as explants or propagules

Establishment of In Vitro Plantlets from Plant Materials

1. When starting with plants, excise nodal sections from the third and fourth nodes from the stem apex with a scalpel (Fig. 1). Stems should be 1-2 cm long and the leaf should be detached. If tubers are the source material, sprouts are removed (usually two sprouts/tuber) by grasping them with forceps and gently bending them until they break away from the tuber.
2. In the laminar flow hood, surface sterilize tissue for 10 min in 0.5% sodium hypochlorite, rinse in sterile H20, and recut the base of the tissue with a scalpel. Place nodal sections or sprouts into individual culture tubes containing MS medium (Fig. 1).
3. Allow growth to 4-6 nodes/stem stage.
4. Test plantlets for virus by ELISA (see protocol below).

ELISA

Test Note. Antibodies and conjugated antibodies against potato viruses and many other plant viruses can be purchased from Agdia, Inc.

1. Prepare the wells of a low-binding ELISA plate by adding 0.1 ml of the antibody stock diluted 1: 1000 in coating buffer to each test well. Coat two wells per sample for each virus to be tested for and additional wells for controls. Incubate in a humidified box overnight at 4 DC or for 4 h at 37 DC.
2. Prepare samples by removing leaves from plantlets in the laminar flow hood. This should be done carefully so as to not damage nodes and to maintain sterile conditions. Grind tissue in 0.01 M phosphate buffer, pH 7.4 and 0.85% saline (PBS) with 2% PVP and 0.1 %

ovalbumin at a 1: 10 dilution (w:v). Positive, negative, and buffer controls should be prepared. Store samples at 4 DC until ready to use.

3. Empty the plate by inverting over a sink and shaking. While still inverted, hit it sharply on several paper towels three to four times. Rinse the plate by filling the wells with PBS and wait 3 min. Repeat the rinsing 2 X more.
4. Add 0.1 ml of the prepared sample to each of two wells on the ELISA plate. Incubate in a humidified box overnight at 4 DC.
5. Repeat step 3.
6. Add 0.1 ml of the conjugated antibody stock diluted 1: 1000 in the sample preparation buffer. Incubate in a humidified box for 4 hat 37 DC.
7. Repeat step 3.
8. Add 0.1 ml of substrate buffer with 1 mg/ml of p-nitrophenylphosphate to each well and monitor for a yellow color reaction. The ELISA plate may be read at a 405 nm wavelength with a spectrophotometer, the positive control OD should be 1.2 to 1.5.

Propagation by Nodal Cuttings

1. In the laminar flow hood, use the large forceps to remove a plantlet from the culture tube and place it on a sterile filter paper.
2. Steady the plantlet with the small forceps, and using either the dissecting scissors or the scalpel, remove the leaves at the distal end of the petiole.
3. Using either the dissecting scissors or the scalpel, sever the main stem above and below each node into sections about 1-2cm in length (Fig. 2). Place one node in each culture tube [illegible]edium with the large forceps.

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Murashige and Skoog Medium (MS) provides all the essential macroelements and microelements. Potassium dihydrogen phosphate serves as a source of phosphate. Microelements like Boron, Manganese, Molybdenum, Copper and Zinc play vital role in plant metabolism

Modified Nodal Cuttings

1. Establish plantlets by nodal cuttings and grow to six-node stage.
2. In the laminar flow hood, make nodal cuttings from the third and fourth nodes. Use a scalpel and forceps to peel away the leaf and petiole.
3. Make a V -shaped cut in the stem to remove bud (Fig. 2).
4. Place modified nodal cutting on MS medium. Transfer to fresh MS medium as shoots grow.

Meristem Excision

1. Establish plantlets by nodal cuttings and grow to six-node stage.
2. Make nodal cuttings from the third and fourth nodes of respective plants.
3. Place 1 cutting under the dissecting microscope at 10-20X magnification, and use scalpel and forcep to peel away protective leaves on bud.
4. Excise meristematic dome plus 1st set of leaf primordia with scalpel, 0.3- O.7mm (Fig. 2).
5. Place excised meristem on Meristem medium. Mark petri dishes into quadrants with one meristem in each. Keep the dish closed whenever possible, seal with Parafilm when full.

Note. Place the petri dish under the microscope to check the placement of the meristem. If growth is not evident in 2-4 weeks, transfer meristem to fresh medium.

6. Grow plantlets from meristems to the six-node stage.
7. Test plantlets for virus by ELISA.

   Note. A final test for virus should follow transplanting plantlets to soil mix and growing to maturity.

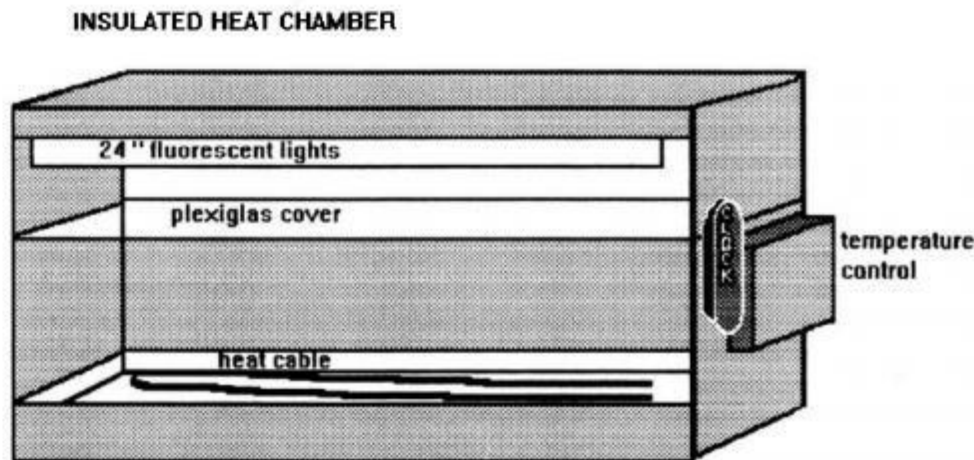

Fig. 3. Heat chamber used for heat therapy of 2-node stage plantlets in culture

Heat Therapy

- Establish plantlets by modified nodal cuttings in culture tubes.
- At the two-node stage, put plantlets in heat chamber at 35 °C with 25 µmol $m^{-2}s^{-1}$ light (Fig. 3), and retain control plantlets at room temperature, 23 °C. Note. We use a 4-h alternating 35°C lights-on and 31 °C lights-off protocol. If a continuous temperature regimen is used, we recommend 35°C and 24 h lights-on.
- Therapy period is for 4 weeks.

  Note. Plantlets should be monitored. If mortality is excessive, the therapy period may be shortened to 2-3 weeks.
- After therapy, test plantlets for virus immediately upon removal from heat chamber. Discard plantlets testing virus positive.
- Excise meristems or modified nodal cuttings as before, and repeat growth to six-node stage.
- Test plantlets for virus by ELISA.

  Note. A final test for virus should follow transplanting plantlets to soil mix and growing to the compound leaf stage.

Results

An example of a data sheet to record experimental information is shown in Fig. 4. Three tables are shown from Sanchez et al. (1991) demonstrating the effectiveness of different treatments on virus

elimination (Tables 1, 2, 3). Treatments include: (1) room temperature, 23 °C, (2) heat treatment/alternating 35°C light and 31 °C dark schedule on 4-h intervals, (3) chemotherapy with ribavirin at 23°C, and (4) chemotherapy with ribavirin at 35/31 °C as in treatment 2. The data indicated a quantitative reduction in virus titer following heat and/or chemical therapy. When virus was not detectable following therapy, propagation by modified nodal cuttings was as efficient as meristem culture in the generation of virus-free plants. As virus titer increased in treated plants, virus elimination was better with meristem excision than with modified nodal cuttings. Modified nodal cuttings had the advantage of more rapid regeneration into plantlets.

TEMPERATURE	

MODIFIED NODAL CUTTINGS	WEEK	# CUT	SURVIVING #	SURVIVING %	# AT 6 NODE STAGE	VIRUS FREE #	VIRUS FREE %	COMMENTS
MERISTEMS								

Fig. 4. Virus elimination data sheet format

Table 1. Effect of therapy on viral titers in potatoes

Tested virus[a]	No. of genotypes with virus level reduced/no. tested[b]	ELISA (A_{405})values			
		MS-RT	MS-HT	MSR-RT	MSR-HT
PVS*	8/8	1.10 ± 0.48[c]	0.46 ± 0.36	0.41 ± 0.34	0.08 ± 0.06
PVX*	1/10	0.22 ± 0.14	0.11 ± 0.18	0.03 ± 0.03	0.02 ± 0.01
PVY*	0/3	0.11 ± 0.12	0.03 ± 0.02	0.09 ± 0.08	0.11 ± 0.16
PVS**	24/25	0.91 ± 0.25	0.31 ± 0.22	0.26 ± 0.24	0.05 ± 0.07
PVX**	7/11	0.57 ± 0.21	0.12 ± 0.20	0.14 ± 0.06	0.03 ± 0.03
PVY**	8/12	0.71 ± 0.28	0.43 ± 0.24	0.56 ± 0.35	0.11 ± 0.13
PLRV**	7/12	0.51 ± 0.29	0.33 ± 0.28	0.40 ± 0.35	0.21 ± 0.12

[a] Virus tested for by ELISA. Genotypes infected with one(*) or multiple(**) viruses.
[b] Highly significant reduction ($P < 0.01$) in virus concentration of treated plantlets compared to control plantlets.
[c] Values represent a mean of 20 plants/treatment/genotype. MS-RT = Murashige-Skoog medium, control plantlets maintained at 23 °C; MS-HT = Thermotherapy plantlets at 35 °C in light/31 °C in dark on 4-h alternating schedule for 30 days, MS medium; MSR-RT = MS medium amended with 20 mg/ml ribavirin at 23 °C; MSR-HT = combined MSR and HT treatment.

Table 2. Effect of treatment and explant propagation on regeneration of potato plantlets

Treatment[a]	Explant propagation (%)[b]	
	Meristem tip	Nodal cutting
MS-RT	42	90
MS-HT	41	90
MSR-RT	45	93
MSR-HT	43	91

[a] MS-RT = Murashige-Skoog medium, control plantlets at 23 °C; MS-HT = Thermotherapy plantlets at 35 °C in light/31 °C in dark on 4-h alternating schedule for 30 days, MS medium, MSR-RT = MS medium amended with 20 mg/ml ribavirin at 23 °C; MSR-HT = combined MSR and HT treatment.
[b] Explants were meristem tips of 0.2–0.4 mm length or a single nodal cutting without the subtending leaflet. Percentiles based on >100 plantlets/treatment.

Table 3. Efficiency of virus elimination from plantlets following therapy based on the effectiveness of the treatment on plantlets and method of explant propagation

Virus	Explant propagation	Titer group of treated plantlets[a]					
		Low		Medium		High	
		Number[b]	%	Number	%	Number	%
PVS	Nodal cutting	131/178	74	33/243	13	0/95	0
	Meristem tip	75/102	74	27/106	45	9/45	20
PVX	Nodal cutting	135/161	84	37/79	47	0/24	0
	Meristem tip	61/72	85	13/29	45	5/19	26
PVY	Nodal cutting	59/128	46	17/126	13	0/39	0
	Meristem tip	30/57	53	15/51	29	4/22	18
PLRV	Nodal cutting	27/62	44	22/184	12	0/10	0
	Meristem tip	27/38	71	34/66	51	3/8	37

[a] Titer groups were based on ELISA (A_{405}) values as follows: Low ≤0.05 OD; Medium 0.05 < OD < 1.00; and High ≥1.00 OD.
[b] Number of plantlets testing at the indicated OD values by ELISA (A_{405}) for virus indicated after therapy but before transplantation and grow-out/number of plantlets tested.