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TISSUE CULTURE
FOR
ELIMINATION OF PATHOGENS

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Rolando Lizarraga, Pilar Tovar, Upali Jayasinghe, John Dodds

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TISSUE CULTURE FOR ELIMINATION OF PATHOGENS

Plant pathogens, such as nematodes, fungi, bacteria, and viruses, can be transmitted from diseased to healthy plants. However, not all cells become infected; the meristematic tissues are sometimes disease-free. It is possible to recover non-infected plants by in vitro meristem culture techniques and to grow them into healthy plants.

This document describes the methods that can be applied to remove pathogens from infected material and to produce "pathogen tested" plants for international distribution and propagation in a potato seed program.

1 NATURE OF PATHOGENS

Plant pathogens include nematodes, fungi, bacteria, rikettsia, mycoplasm, viruses and viroids. They are contageous and can be transmitted from diseased to healthy plants. In potatoes until now no reports indicate rikettsia as a pathogen.

The relative size of these pathogens varies greatly. Among the plant pathogens, nematodes are the largest and can be seen easily with a stereo microscope. Viruses and viroids are the smallest and one needs an electron microscope to visualize them.

The occurrance of a disease does not solely depend on the presence of a host and the pathogen. Environmental conditions especially humidity and temperature play an important role in the development of a disease. Thus a disease can be defined as a product of the interaction of the host, the pathogen and the environment. This concept of a disease is known as the "disease triangle".

The distribution of the different pathogens within a diseased plant also varies greatly. Pseudomonas solanacearum, potato leafroll virus, and mycoplasma are restricted to the vascular tissue of a plant. Erwinia carotovora, and potato virus X (PVX), invades both vascular and non-vascular plant tissues. Not all cells in a diseased plant are infected with pathogens. The meristematic tissues of root and shoots of an infected plant are sometimes free of viruses. In certain cases, such as PVX and tobacco rattle virus in potatoes, only the apical dome and the first young primordial leaf are free of viruses. The exact reason for this is not known, however, it is believed that one or all the following factors are responsible.

- High metabolic activity: Viruses replicate by taking over the host metabolic pathways. Due to the high metabolic activity in these cells viruses are unable to take over control of the host biosynthetic machinery.

- Lack of vascular system: Viruses spread rapidly through the vascular system. Phloem restricted viruses (PLRV) cannot invade the meristematic tissues due to the absence of cell differentiation. In this meristematic region, viruses which infect non-vascular tissues spread from cell to cell through the plasmodesmata. This is a slow process which makes it relatively difficult for viruses to completely infect the rapidly dividing cells.

- High auxin concentration: Plant meristematic tissues have a higher auxin concentration than tissues from the other plant regions. These auxins have been reported to inhibit the replication of viruses.

Since meristematic tissues are sometimes free of pathogens, it is possible to recover non-infected plants by in vitro meristem culture techniques and to grow them into healthy plants.

Thermotherapy at elevated temperature (37 °C) does not eliminate the potato spindle tuber viroid (PSTV). PSTV consists of a single-stranded, ring-shaped RNA that is twisted in the form of a supercoil. In this form it is resistant to nucleases. Elevated temperature, far from decreasing the concentration of the viroid, favors its multiplication (Sanger and Ramm 1975). Therefore, a first test for PSTV should be carried out at the end of the thermotherapy period.

A method that permits the eradication of PSTV (Lizarraga et al. 1980) is based on the observation that in plants grown at low temperatures, the viroid concentration is low. In an experiment, plants were grown at 8°C for 4 months. Then apical domes were excised. 30 % of the plantlets regenerated were free of PSTV, even in the second tuber generation. A clear (negative) relationship between meristematic explant size and eradication success was observed (Lizarraga et al. 1982).

This method, however, is not suitable as a routine technique, since it is time consuming and costly. It may be useful in specific cases in which a valuable clone is heavily infected and no free material available.

Experiments carried out with different virus host systems have shown that treatment of plants with elevated temperatures (thermotherapy) leads to a reduction in virus concentration (titre) in the plant (Kassanis 1957; Quak 1977). Different reasons have been given to explain this phenomenon; probably not one alone, but a combination of several may be the cause for reduction in virus titre. These can include competition for sites of synthesis of nucleic acids and proteins between the fast

dividing host cells and the virus particles, which may lead to a change in the balance between synthesis and degradation of virus particles. Also the nucleic acid of the virus, the carrier of its genetic information, is usually protected from attacks by degrading enzymes because of a coat, made of many protein subunits. At elevated temperatures the linkage between these subunits becomes weaker, temporary holes may open and permit the attack of nucleases, leading to inactivation of virus and decrease of virus concentration.

Thermotherapy has been applied to dormant potato tubers. A reduction of virus concentration, mainly of potato leafroll virus (PLRV) has been observed. However, elimination was not achieved except for PLRV.

Thermotherapy applied to the whole plant as well as to sprouted tubers followed by meristem culture has been successfully used for elimination of many viruses in potato (Stace-Smith and Mellor 1970; Pennazio and Redulfi 1973).

In the standard procedure, best results have been obtained when the plant is decapitated before introduction into thermotherapy and axillary buds grown while undergoing heat treatment. A daily temperature regime of 36 °C for 16 hours and 30 °C for 8 hours and continuous light of high intensity (10,000 lux) improved elimination rates. Plants are kept under these conditions for 4 weeks. From axillary buds as well as apical buds meristems are isolated and cultivated, as shown in Section 6.

3   CHEMOTHERAPY

As an alternative to thermotherapy, chemotherapy has been tried in pota-
to. A nucleoside analogue, Virazole, known for its broad spectrum
against animal DNA and RNA viruses has shown variable results when app-
lied to the potato plant as spray or in hydroponic culture followed by
meristem culture. Preliminary results show some promise when culturing
excised meristem tips in the presence of 100 ppm Virazole in the medium.

However, some reports indicate that antiviral chemicals may cause muta-
tions in the plant. Therefore, at the present time, thermotherapy is
definitely the preferred method of pre-treatment prior to meristem cul-
ture.

If the surface of plant material is contaminated by pathogens or sapro-
phytes, some of the contaminants quickly grow and ca kill the plant.
Most surface contaminants, such as bacteria and fungi, can be eliminated
by surface sterilizing the plant material with a suitable sterilizing
agent.

Surface sterilizing agents are normally applied for 10 - 15 minutes.
Under aseptic conditions, the sterilizing solution is then removed and
the plant material washed 3 or 4 times for 5 minutes each time by agita-
tion in sterile distilled water. Washing is important to remove excess
sterilizing agent which inhibits plant growth.

Ethanol (Alcohol). Alcohol is a common surface sterilizing agent for
killing bacteria and fungi, and is often given as a brief wash prior to
applying other surface sterilizing treatments. It has a low surface
tension and can easily penetrate between leaf hairs and wet the surface
of the plant.

Sodium or calcium hypochlorite. Plant material may also be surface ste-
rilized with an aqueous solution of either sodium hypochlorite (NaOCl)
or calcium hypochlorite (Ca[OCl]). The calcium salt is preferred as it
is less phytotoxic. Many laboratories use a household bleach such as
Clorox. These commercial products usually contain 5.25 % NaOCl as the
active agent. When diluted with water (1 part bleach : 9 parts water),
the final sterilizing solution should contain not less than 0.5 % NaOCl.

Because of complete dissociation, hypochlorite has relatively little activity at pH over 8.0, and it is much more effective by buffering the solution at about pH 6.0.

Freshly excised tissue, completely immersed in the hypochlorite solution, is surface sterilized after an exposure of 10-15 minutes. Following the hypochlorite treatment the plant material must be thoroughly rinsed with several changes of sterile distilled water to remove the disinfectant completely.

Mercuric chloride. Mercuric chloride ($HgCl_2$) has been used as a disinfectant, although it is extremely toxic. A solution of mercuric chloride is volatile at room temperature and can cause mercury poisoning. Therefore, WE DO NOT ADVISE ITS USE AS A STERILIZING AGENT !

Bactericides and fungicides. With material that is heavily contaminated some researchers recommend washing in a commercial bactericide/fungicide mix prior to surface sterilization. It should be remembered however that this treatment has no effect on systemic infections. Section 3 gives a list of bactericides and fungicides together with information about their use.

5   ANTIBIOTICS

Although employed routinely in animal cell cultures to prevent bacterial contamination, antibiotics have not been widely used in plant tissue cultures. Early botanists were aware that these natural products may alter the growth and development of plant tissues cultures in vitro (Gautheret, 1959; Butenko, 1964). In fact, plant tissues are sensitive to antibiotics and show variable responses according to their genotype.

Most tissue culture specialists do not rely on antibiotics to remove superficial contaminants from explants. The drugs are expensive and no one is effective against all possible types of contaminating organisms. Antibiotics are only employed where microorganisms are difficult to eliminate by other means.

The following antibiotics or combinations of antibiotics have been successfully applied:

- Gentamycin
- Rifampicin
- Nystatin + Carbenecillin
- Gentamycin + Amphotericin B
- Vancomycin-HCl + Mycostatin
- Streptomycin + Carbenecillin

Toxicity has been reported to be caused in some cultures by:

- Penicillin
- Streptomycin
- Bactericin
- Sparsomycin

The active growing point of the plant shoot is the meristem.   It is a small region composed of rapidly dividing (meristematic) cells.

The dome of a shoot apical meristem contains the truly meristematic cells and is surrounded by leaf primordia and primary leaves.   Since the more differentiated vascular tissues are found distant from the meristem (towards to older tissue of the stem), the vascular elements of the leaf primordia are still incipient, and have not yet made contact with the main strand of the vascular system in the stem.   Therefore, virus particles, which may be present in the vascular system, can reach the meristematic region of the apex only through cell to cell movement; a slow process.   This is one of the main reasons why in a virus infected plant, virus concentration decreases acropetally toward the meristem of both the apical and the axillary buds.

Isolation of the apical portion, called the meristem tip, under aseptic conditions and its culture on an adequate aseptic nutrient medium, leads to the development of plantlets.   This developmental sequence, in principle, follows a pattern similar to that in the normal plant growth. The cells of the meristem divide and the differentiation of new tissues continues.   The nutrition of the excised portion by the plant is supplied by the artificial medium.   This technique, called meristem culture, was first applied for virus erradication some 30 years ago by Morel and Martin (1952) on Dahlia, and leads to pathogen-free plants.

The aseptic dissection of the meristem is a delicate process and requires many hours of practice. The sequence of dissection is shown photographically (Figure 3) and is carried out as follows.

Stems are cut from the plant that has just undergone thermotherapy into segments each containing one node with its axillary bud. The leaves are carefully removed. the stem segments are disinfected for 30 seconds in 70 % alcohol, followed by 2.5 % calcium hypochlorite for 15 minutes. Then the stems are washed four times 5 minutes each with sterile distilled water to remove excess hypochlorite.

Under a binocular dissecting microscope, the leaflets surrounding the growing point are removed until only the apical dome and a few leaf primordia remain. The dome and two leaf primordia are excised and transferred to meristem culture medium (see Appendix). The excised apical dome is transferred weekly to fresh medium. After 6-8 weeks, the small plantlets are subcultured for further growth and micropropagation (Espinoza et al. 1984).

After regeneration from the cultured meristems, plants are tested (indexed) to detect any persisting virus infections. See the following references in Section 9:

Fribourg, C., Nakashima, J. 1981 – Latex test
Salazar, L.F. 1981 – PSTV detection
Salazar, L.F. 1982 – Virus detection
Salazar, L.F. 1983 – ELISA

In vitro material can be conserved in culture indefinitely when suffi-
cient care is taken to avoid contamination, and transfer to fresh media
is made at appropriate intervals. Pathogen-tested in vitro plantlets
can be kept as base collections for propagation in a seed program.

For short-term maintenance, plants are grown in tubes on propagation
medium B (see Appendix). The tubes are sealed with autoclavable plastic
caps. These are better than cotton wool plugs that are easily penetra-
ted by air-born fungal spores and insects.

The growth rate of the plants depends on incubation temperature, medium
composition, and genotype.

Long-term storage media C and D (see Appendix) exert an osmotic stress
on the plantlets and can be used for storage at 25 °C; the stress redu-
ces the growth rate and causes short internodes. Many nodes are thus
available when propagation of the stored material is initiated. Mate-
rial stored under these conditions normally needs to be transferred once
a year only.

When using Medium C, the temperature can be lowered to 8 °C. This sig-
nificantly reduces the growth rate of the plantlets and transfers are
only required once every 2 or 3 years.

It is possible, therefore, to maintain a germplasm collection under the
storage conditions outlined above. When a request is received for ex-
port of a particular pathogen tested genotype, it can be removed from
storage, micropropagated, and distributed in vitro.

All media used for this work are based on the salts of Murashige and Skoog (1962). The salt stock solutions are normally prepared in four separate parts:

- Salts
- $MgSO_4$
- Iron
- Vitamins

<u>Salts</u> stock solution: dissolve each salt in 200 cm^3 distilled water

-	NH_4NO_3	35	g
-	KNO_3	40	g
-	$CaCl_2.2H_2O$	9	g
-	KH_2PO_4	3.5	g
-	H_3BO_3	0.1	g
-	$MnSO_4.4H_2O$	0.5	g
	$(MnSO_4.H_2O$	0.4	g)
-	$ZnSO_4.7H_2O$	0.2	g
	$(ZnSO_4.H_2O$	0.1	g)
-	KI	0.02	g
-	$Na_2MoO_4.2H_2O$	0.005	g

Dissolve 5 mg (0.005 g) of the following salts together in 10 cm^3 of water; add 1 cm^3 of this solution to 200 cm^3 water for the stock solution

- $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$

Mix the ten individual salt solutions together to make 2000 cm^3 of the salt stock solution.

MgSO$_4$ stock solution

-    MgSO$_4$.7H$_2$O   3.7 g in 100 cm^3 distilled water

Iron stock solution

-    Na$_2$EDTA   0.75 g
-    FeSO$_4$.7H$_2$O   0.55 g

Dissolve FeSO$_4$.7H$_2$O in 20 cm^3 distilled water; Na$_2$EDTA in 20 cm^3 warm distilled water. Mix the solutions, cool, and make up to 100 cm^3 with distilled water.

Vitamin stock solution

-    Thiamine HCl   40 mg in 100 cm^3 distilled water

Medium preparation. Prepare 1 liter (dm^3) of the Murashige-Skoog basic medium by mixing the stock solutions with Inositol in the following proportions:

Salts	100 cm^3
$MgSO_4$	10 cm^3
Iron	5 cm^3
Vitamin	1 cm^3
Inositol	100 mg

Add the relevant hormones and sucrose (see below), and autoclave the medium either with or without agar for 15 minutes.

Additions to the basic MS medium for preparation of specific media types

Medium	+	additions		
A (meristem)	0.25	ppm	Gibberellic acid
	2.00	ppm	Ca Pantothenic acid
	3.0	%	Sucrose
	0.6	%	Agar
B (propagation)	0.25	ppm	Gibberellic acid
	2.00	ppm	Ca Pantothenic acid
	3.0	%	Sucrose
	0.8	%	Agar
C (storage)	4.0	%	Mannitol
	3.0	%	Sucrose
	0.8	%	Agar
D (storage)	4.0	%	Mannitol
	0.5	%	Sucrose
	0.8	%	Agar

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