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THERAPY CYCLING TO ELIMINATE HIGH-TITERED, MULTIPLE VIRUS INFECTION *IN VITRO* POTATO PLANTLETS

Iswari S. Dewi[1] and Steven A. Slack[2]

ABSTRACT

A protocol for treatment of in vitro potato planlets to eliminate systemic viruses was established. Efficiency decreased, however, for selected genotypes when a virus was high-titered and/or multiple viruses existed. Modified nodal cutting (Mncs = ≤ 0.5 mm) of the speciality potatoes 'Purple Fingers' infected with PVS, PVY, and PLRV and ' All Red" infected with PVS, PVx, and PLRV were established on a medium containing antiviral Ribavirin (20 mg/l) and subjected to 4-6 weeks thermotherapy. This cycle was repeated for clones testing positive by ELISA for more than one virus. After one therapy cycle, 14% of 'Purple Fingers' and 8% of 'All Red' plantlets tested were free from PVS, PVX, PVY, and PLRV. System inefficiency was primarilty due to low elimination of PLRV which were 14% from 'Purple Fingers' and 13% from 'All Red'. Following the second cycle, 95% of 'Purple Fingers' and 64% of 'All Red' plantlets were virus free with 100% and 69% freedom for PLRV, respectively. A second therapy cycle was recommended to enchance system efficiency to eliminate recalcitrant viruses.

ABSTRAK

Efisiensi dari standar protokol untuk mengeliminasi virus kentang secara *in vitro* menurun, terutama untuk genotipe tertentu yang mengandung kadar virus yang tinggi dan/atau telah terinfeksi bermacam virus. Modifikasi 'nodal cutting' (≤ 0.5 mm) dari 'specialty potato' yaitu 'Purple Fingers' yang terinfeksi dengan PVS, PVY, dan PLRV serta 'All Red' yang terinfeksi dengan PVS, PVX, dan PLRV ditanam pada media yang mengandung zat antivirus Ribavirin (20 mg/l) kemudian diterapi selama 4-6 minggu dengan suhu tinggi (31°C gelap/35°C terang selama 4 jam bergantian). Terapi yang sama diulang lagi untuk kedua kalinya pada genotipe yang masih mengandung virus menurut pemeriksaan ELISA. Pada akhir terapi pertama, 14% dari 'Purple Fingers' dan 8% dari 'All Red' dapat dibebaskan dari PVS, PVX, PVY dan PLRV. Ketidakefisienan terapi terutama disebabkan karena adanya PLRV yang sukar untuk dieliminasi, yaitu hanya 14% dari 'Purple Fingers' dan 13% dari 'All Red'. Dengan terapi kedua, 95% dari 'Purple Fingers' dan 64% dari 'All Red' dapat dibebaskan dari virus-virus yang menginfeksinya, yakni 100% 'Purple Fingers dan 69% 'All Red' dapat dibebaskan dari PLRV. Terapi berulang direkomendasikan untuk mempertinggi efisiensi sistem yang digunakan untuk mengeliminasi virus kentang secara *in vitro*.

1) Researcher at Cell Biology Laboratory, Research Institute for Food Crops Biotechnology (RIFCB/BalitBio), Bogor - Indonesia
2) Henry-Mildred Uihlein Professor at the Department of Plant Pathology, Cornell University, Ithaca, NY. 14853-USA.

INTRODUCTION

Speciality potato varieties have special characteristics such as unique skin colors, flesh colors, and/ or tuber shapes. It is used mainly as decoration ('garnish') in salads or on special occasions (as an 'offerings'). They were grown, asexually propagated, many generations in the field and were observed to have severe virus symptoms such as mosaic, leaf necrosis or leafroll. Until now there has been little, if any, good quality seed material available. There is a need to provide clean seeds for those interested in producing these varieties.

Researchers have developed procedures for eliminating systemic infection that persisted in the vegetative parts caused by viruses. The success of meristem-tip culture, chemotherapy and thermotherapy and then combinations of heat and chemical treatment in eliminating certain viruses have been reported (Mellor and Stace-Smith, 1977; Quack, 1977; Bhojwani and Razdan, 1983; Griffiths *et al.*, 1990).

Meristem-tip culture is the first procedure established as an efficient technique for eliminating viruses. Although it seems to be a simple technique, meristim-tip culture has limitations, such as the long regenartion time (approximately 6-8 months), low survival rate and require high degree of technical expertise for exicising the mreistem under microscope (Mellor and Stace-Smith, 1977; Quack. 1977; Bhojwani and Razdan, 1983).

Chemotherapy and thermoterapy procedures have been reported to have some encouraging results in virus elimination (Baker, 1962, Hollings, 1965; Kelin and Livingstone, 1982; Bhojwani and Razdan, 1983; Roca, 1983). A number of chemicals have been found as an antiviral agent capable of eliminating or reducing the multiplication of viruses. Ribavirin (trade mark 'Virazole') a nucleoside analogue has been shown to be the most active chemical in eliminating viruses. Ribavirin has been reported to act as inhibitor of virus (i.e. PVM, PVS, PVX) replication in potato plantlets *in vitro* (Klein and Livingstone, 1982, Wambugu *et al.*, 1985; Griffiths *et al.*, 1990). However, the efficiency of Ribavirin alone decreased when plant infected with a high-titered and/or having multiple virus (Griffiths *et al.*, 1990).

Thermotherapy has been effectively used for a long time to obtain virus-free plants of diverse plant species. Hot air temperature treatment has given better elimination of viruses and better survival of the host in actively growing shoots and proved to be better for dormant bud compared to other heat treatment procedure, such as hot water treatment (Hollings. 1965). The basic principle behind heat elimination of viruses is that at temperature higher than ambient temperature many viruses in plant tissues are partially or completely inactivated with little or no injury to the host (Baker, 1962). A major limitation of thermotherapy for virus elimination is that not all viruses are sensitive to the treatment, while continuous exposure to the high temperature proves to be harmful to the host tissues (Bhojwani and Razdan, 1983). Moreover, Roca (1985) explained that heat treatment did not always eliminate most viruses, but it reduced virus multiplication and translocation in the plant.

Combination of heat and chemical treatment for virus elimination in *in vitro* potato plantlets to eliminate systemic viruses has been standardized (Griffiths *et al.*, 1990). The study has been able to demonstrate that virus elimination could be obtained without requisite of meristem-tip excision step. Using nodal cutting combined with Ribavirin (20 mg/l) and a continuous alternating 4 hours 31°C dark/4 hours 35°C light regime, they obtained virus-free potato plants from PVS, PVX, and PVM, except for PVY and PLRV. However, the efficiency of this standard procedure decreased when a virus was high-titered and/or multiple virus existed.

The objective of this study was to improve efficiency of standard procedure for treatment of *in vitro* potato plantlets when high- titered multiple viruses existed.

MATERIALS AND METHODS

Plant Material

Tubers of two specialty potato varieties, 'Purple Fingers' (slender tuber shape, purple color in flesh and skin) and 'All Red' (round tuber shape, red color in flesh and skin) were obtained from a grower in Upstate New York. Tubers were harvested from field grown plants exhibiting multiple and severe virus symptoms. All plants appeared to show similar disease symptoms.

Tubers were washed and surface-sterilized by immersing in 0.5% Sodium Hypochlorite solution for ten minutes and rinsed with deionized sterile water. They were allowed to develop green sprouts under light at 23°C with a 16-h day length of 2000 lux. When sprouts were about 10-15 mm in length, they were excised from the tubers. Under sterile condition (laminar air flow) sprouts were immersed in 0.5% Sodium Hypochlorite solution for 10 minutes and rinsed three times with deionized sterile water. The base of the sprouts were discarded and only the top portion of each sprouts were placed in MS medium and grown at 23°C with 16-h day length of 2000 lux.

Plantlets from tubes showing no visible contamination were multiplied by nodal cutting (one axillary bud each with subtending leaflets excised) to establish mother plants *in vitro*. Excised lower leaves from establihed mother plants were tested for viruses by Enzyme Linked Immuno Sorbent Assay (ELISA). For ELISA, the peroxidase system was used. Kits were obtained from Agdia Inc., Elkhart, Indiana 46514, USA. 'Purple Fingers" was found to be infected with PVS, PVY and PLRV whilst 'All Red' was infected with PVS, PVX, and PLRV.

Culture Medium

For propagation of *in vitro* plantlets, MS medium (Murashige and Skoog, 1962) was used and consisted of the following : 43 g l^{-1} MS salts, 0.17 g l^{-1} NaHPO4, 0.4 g l^{-1} Thiamine. HCl, 0.1 g l^{-1} Myo- inositol, and 30 g l^{-1} Sucrose. The pH was adjusted to 6.0 using 1 M KOH prior to the addition of 2.0 g l^{-1} Gelrite Gellan Gum (Kelco, San Diego, CA.) as a solidifying agent.

For chemotherapy treatment, MS medium was amended with Ribavirin (Serva, Westburry, NY.) at 20 mg l^{-1} and IAA at 1 mg l^{-1} designated as MSV medium. After microwaving for 15 minutes, the medium was dispensed at a rate of 10 ml per test tube (size of 20 x 150 mm). The filled culture tubes were autoclave-sterilized for 18 minutes at 121°C of 18-20 psi.

Experimental Treatment

In vitro plantlets of the two varieties were the source materials (mother plants) for modified nodal cuttings. According to Roca (1985) *in vitro* very small explant such as shoot-tip (0.1-0.2 mm) was quite slow and often led to callus formation. Modified nodal cutting (Mnc) was a minute axillary bud with a very tiny bits of stem tissues that made the size of ≤ 0.5 mm for the whole explant. Explants were cut under a dissecting microscope. We used Mnc considering that the explant would be small enough to eliminate viruses but large enough to be able to develop into a complete normal plant.

After Mncs developed into normal plantlets in MSV medium (2-3 cm in shoot with normal root formation) they were subjected to 4-6 weeks heat therapy in an uninterupted alternating 4-h 31°C dark/ 4-h 35°C light period. Following the therapy plantlets were tested quantitatively (OD at A_{405}) for appropriate viruses by ELISA. The plantlets were consider virus-free or negative when the OD ≤ 0.05 and virus infected or positive when the OD > 0.05.

Mncs were excised from plantlets testing negative at the conclusion of the treatment (either one or two cycles) and regenerated at 23°C with 16-h day length of 2000 lux. One copy of plantlets was transplanted in the greenhouse and retested for viruses by ELISA when compound leaf morphology was observed (approximately at 3-4 weeks). The plantlets were confirmed as virus-free (VF) when plants from greenhouse grow-out had OD ≤ 0.05 from their specific viruses.

Selection for the second cycle was based on a cutting derived from a plantlet that was not VF at the end of the first therapy. The Mnc should be regenerated to a plantlet in a reasonable length of time (less than or in 4 weeks in MSV medium).

Since poor regeneration of those Mnc were observe when they placed directly in MSV medium after being out from the first therapy, they were placed in nursing medium consisting the following : 4.3 g l^{-1} MS salts, 0.1 g l^{-1} Myo-inositol, 30 g l^{-1} Sucrose, 0.1 g l^{-1} Gibberelic Acid, 2.0 mg l^{-1} Thiamine HCl, 2.0 mg l^{-1} Glycine, 0.5 mg l^{-1} Pyridoxin. HCl, 0.5 mg l^{-1} Nicotinic Acid and 0.04 mg l^{-1} Kinetin. The pH was adjusted to 6.0 using 1 M KOH prior to the addition of 7.0 Difco Bacto Agar as a solidifying agent.

The Mncs remained in the nursing medium until root initials were observed (approximately 5 days). The Mncs then were moved to tubes of MSV medium, established as plantlets and recycled into a second therapy cycle of Ribavirin and heat identical to the first therapy.

Following the second cycle of therapy, plantlets were again tested quantitatively (OD at A_{405}) for appropriate viruses by ELISA. Plantlets that testing negative were treated in the same manner as those testing negative in the first cycle. Plantlets that still testing positive for one or more viruses were discarded.

RESULTS AND DISCUSSION

The standard procedure which combined nodal cutting, heat, and chemical therapy of *in vitro* plantlets was indeed an efficient system to eliminate systemic viruses as demonstrated by Griffiths *et al.* (1990). However, in our study the efficiency of this standard procedure decreased when a high-titered virus and/or multiple virus exist as shown by ELISA (Table 1).

After one therapy cycle, elimination of PVS, PVY, PVX and PLRV were achieved. Table 1 showed that clone with PVS reduced 94% in 'Purple Fingers' and 47.4% in 'All Red', while 86% of 'Purple Fingers' and 94.7% of 'All Red' could be freed from PVY and PVX, respectively. There were only 14% of 'Purple Fingers' and 8% of All Red' plantlets testing free from all viruses. The system inefficiency was primarily due to low elimination of PLRV; *i.e.* only 14% from 'Purple Fingers' and 13% from 'All Red' were free from PLRV.

Table 1. Impact of multiple therapy cycles on elimination of potato viruses in plantlets

Clone	Therapy Cycle	No. of Plantlets	% PVS	PLRV	PVY	PVX	Plantlets Virus Free All Viruses
Purple Fingers	1	50	94.0	14.0	86.0	-	14.0
	2	21	95.2	100.0	100.0	-	95.2
All Red	1	38	47.7	13.2	-	94.7	7.9
	2	52	96.2	69.2	-	96.2	63.5

The low elimination of PLRV was clearly different from the high elimination of PVS, PVY and PVX in both genotypes. Roca (1985) explained that viruses vary in their difficulty to eliminate, those restricted to the phloem were the most difficult to eliminate, except by meristem-tip culture. He made a list of potato viruses according to increasing difficulty of elimination via meristem-tip culture as followed : PLRV, PVA. PVY, PVM, PVX, PVS and PSTV. Moreover, eliminating disease depended on several interrelated factors, such as the virus to be eliminated, the size of the explant to be cultured, and physical or chemical treatment applied to the infected plants or to the culture.

In the case of PLRV, Griffiths *et al.* (1990) using nodal cutting and only one cycle of heat and Ribavirin were unable to obtain VF plantlets from PLRV. They assumed that the difference in response may be due to the fact that, in contrast to the other virus, PLRV is a luteovirus confined to phloem tissue and, hence, the site of replication, transciption and translation may differ. In our study, we could obtain PLRV-free plantlets in the first cycle (Table 1), because we use smaller explants than those in Griffiths *et al.* (1990). Therefore, a smaller or less tissues with a lesser virus reservoir were exposed to the system. However, the addition of the second therapy cycle has been able to eliminate 100% and 69% of PLRV in 'Purple Fingers and 'All Red', respectively (Table 1).

Table 2. Impact of multiple therapy cycles on titer of potato viruses in infected plantlets

Clone	Therapy	No. of	Virus		Titer in		Infected		Plantlets	
	Cycle	Plantlets	PVS		PLRV		PVY		PVX	
			No.	A405	No.	A405	No.	A405	No.	A405
Purple Fingers	0	50	50	0.8	50	1.6	50	13	-	-
	1	50	3	0.1	43	0.7	7	0.9	-	-
	2	21	1	0.1	0	0.0	-	-*)	-	-
All Red	0	38	38	1.0	38	1.9	-	-	38	1.1
	1	38	20	0.3	33	0.6	-	-	3	0.2
	2	52	2	1.6	16	0.5	-	-	2	0.4

No. = Number of plantlets
A_{405} = Virus titer (OD at A_{405})
*) = No plantlets were regenerated from plantlets that were still infected with PVY after first cycle.

Table 2 showed that virus titer in infected plantlets was reduced greatly in the first cycle, but it was not very different in the second cycle. It was interesting that in 'All Red' the PVS titer in infected plantlets increased from pretreatment level. The same phenomenon was also observed by Griffiths *et al.* (1990) that virus titer could increase to or even more than pretreatment level after subculturing the plantlets into MS medium. In our study, there was one more Elisa done to confirm virus-free before releasing the VF plantlets.

Following the second therapy, plantlets from both genotypes showed a greater reduction in virus titer. Reduction of virus titer after the completion of the therapy was vary for both genotypes. This therapy cycling system could eliminate multiple virus infection in 'Purple Fingers' and 'All Red' satisfactorily. There were reduction as high as 94% and 98.7% of PVS titer (Figure 1); 98.5% of PVY titer and 98.2% of PVX titer (Figure 2); 92.6 % and 99.4% of PLRV titer (Figure 3) in 'Purple Fingers and'All Red', respectively. It was clear that the addition of the second therapy cycle enhanced the ability of the standard procedure to obtain high percentage of VF plants from *in vitro* plantlets infected with multiple systemic virus. Therefore, the percentage of plantlets testing virus-free increased 8% to 63% for 'All Red' and from 14% to 95% for 'Purple Fingers' (Table 1).

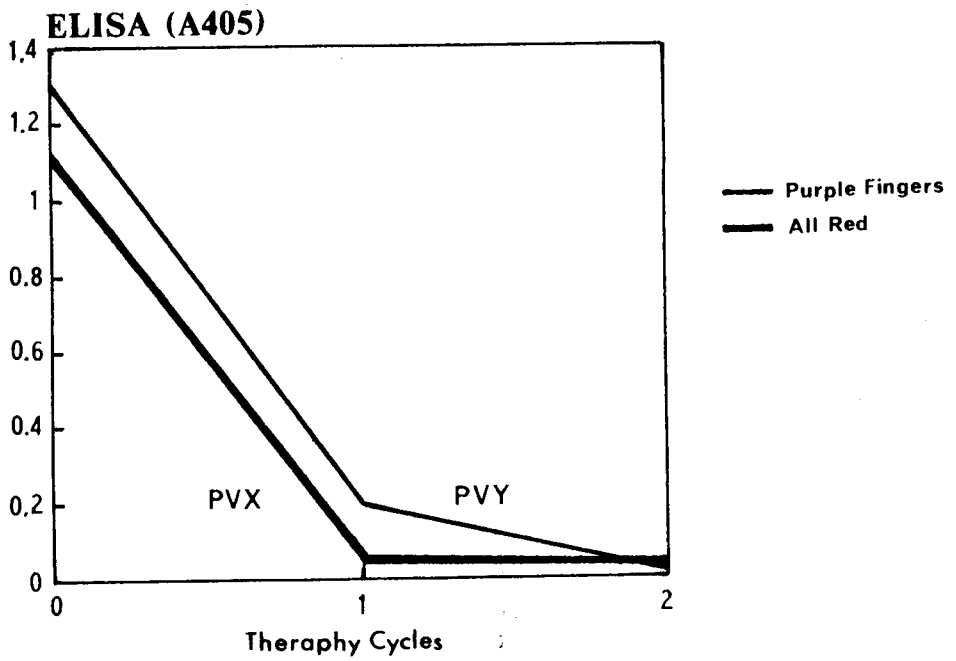

Figure 1. Virus titer of PVX in 'All Red' and PVY in 'Purple Fingers' plantlets following one or two therapy cycles

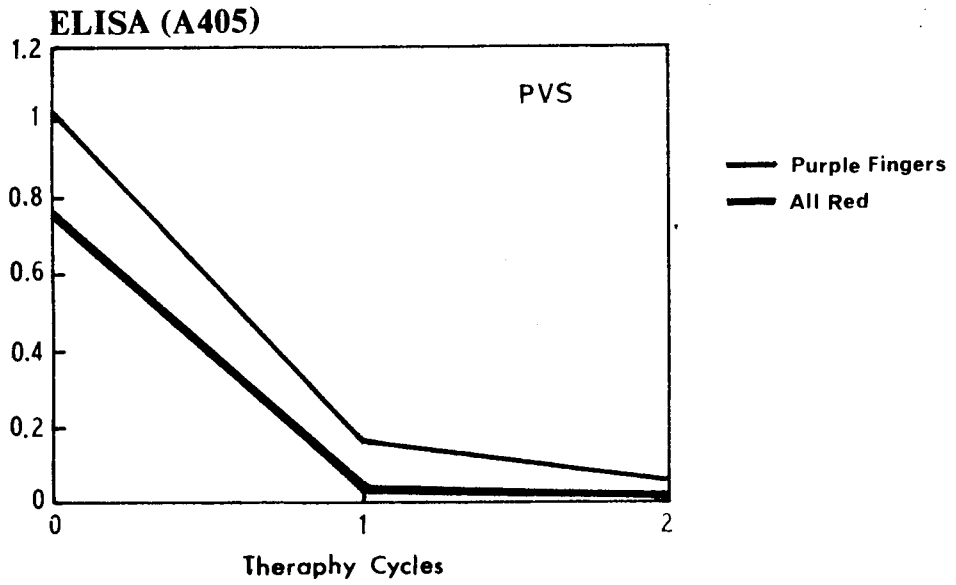

Figure 2. Virus titer of PVS in 'All Red' and 'Purple Fingers' plantlets following one or two therapy cycles

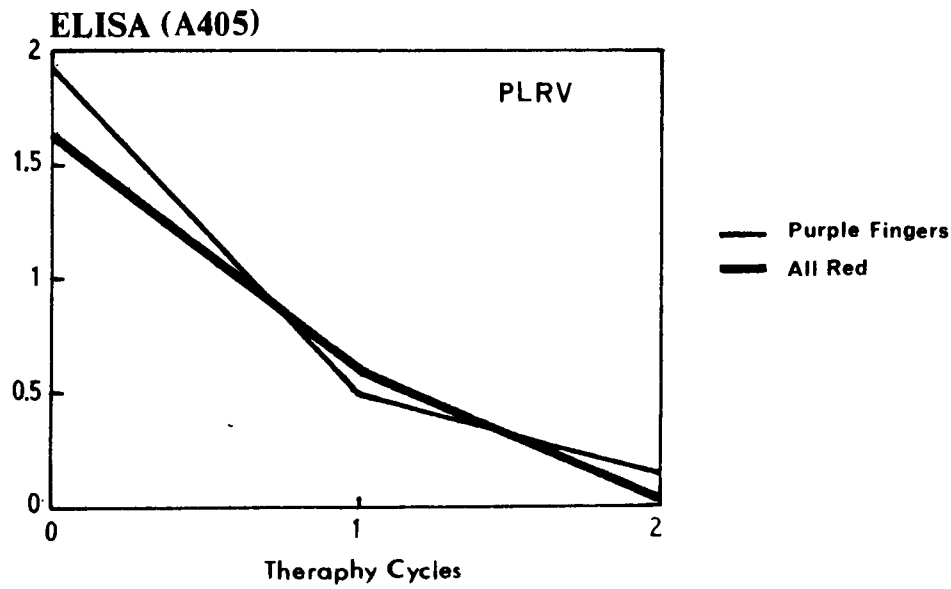

Figure 3. Virus titer of PLRV in 'All Red' and 'Purple Fingers' following one or two therapy cycles

The differences in obtaining VF plants between genotypes were also observed by Griffiths et al. (1990) while testing 12 genotypes. Different response may be due to how the virus interact with the host.

From a practical standpoint, this therapy cycling system is attractive especially for elimination of high-titered and/or multiple systemic virus. Compared with approximately 6-8 months in the case of meristem-tipe culture, this therapy cycling system could provide virus-free plants faster. The time required to produce virus-free plant from plant with virus is approximately 3 months. Moreover, the effectiveness of meristem-tip culture was reduce when plantlets were infected with more than one virus (Mellor and Stace-Smith, 1977). Last but not least, this therapy cycling system does not require a specific technical skill for excising the modified nodal cutting as that with meristem-tip culture.

In summary, the approach taken in this study has successfuly demonstrated that utilizing smaller explant called 'modified nodal cutting' combined with Ribavirin at 20 mg/l and uninterupted 4-h alternating 31°C dark and 35°C light period for two cycles enabled us to eliminate recalcitrant viruses, high- titered viruses, and multiple virus infection in specific genotypes. This approach is especially important since it could eliminate Potato Leaf Roll Virus (PLRV) in high percentage.

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