Short title: Meristem-tip culture for virus elimination in potato

An efficient protocol of chemo-cum-thermotherapy for elimination of potato (*Solanum tuberosum* L.) viruses by meristem-tip culture

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ABSTRACT

With the objective to develop an efficient protocol for virus elimination from potato clones, meristem-tip culture after chemotherapy, thermotherapy or chemo-cum-thermotherapy was attempted in a number of potato genotypes infected of PVX and PVS, the two most difficult viruses to eliminate. Chemotherapy was given by culturing the nodal cuttings from the infected plantlets for 5 weeks on MS medium containing 20 mgl^{-1} of ribavirin. For thermotherapy three weeks old in-vitro plantlets raised from nodal cuttings on MS medium were incubated at 37±1°C for 2 weeks. Chemo-cum-thermotherapy included chemotherapy for 3 weeks followed by thermotherapy of the same plantlets on same medium for 2 weeks. Immuno-electron microscopy of the mericlones so raised clearly showed that chemo-cum-thermotherapy of the infected plants prior to mericloning was the best option for eliminating PVX and PVS as this treatment proved effective in freeing all accessions from these viruses. On the other hand chemotherapy alone was completely ineffective and thermotherapy was effective in a few genotypes. It is concluded that chemo-cum-thermotherapy protocol developed in the present study can be effectively used for cleaning a wide range of genotypes for all viruses by meristem-tip culture in potato, and probably also for other vegetatively propagated plant species.

Key words: Virus elimination, Potato viruses, Mericloning, *Solanum tuberosum*

Abbreviation *MS* Murashige and Skoog (1962)

Potato seed production involves vegetative multiplication of the virus free genetic stocks. Worldwide, potato cultivars as well as elite accessions of potato gemplasm are conserved through vegetative propagation in field genebanks or in-vitro repositories (Lizarraga *et al.* 1989, Gopal and Gaur 1997). Vegetative propagation, however, also propagates viruses, and other endogenous pathogens. Viruses are systemic and their accumulation over time degrades stock plants causing significant reduction in yield, and in extreme cases may also lead to the loss of valuable genetic stocks. As early as 1952, Morel and Martin reported that virus-free dahlia could be isolated by in-vitro culture of the apical meristem of an infected plant, and the resultant plants were also genetically identical to the mother plant. Since then this technique has been applied to a number of vegetatively propagated crop species (Mellor and Stace-Smith 1977, Quak 1977) including potato, which is host to a number of viruses. At least 40 viruses, some with several characterized strains, are known to affect potato crops (Jeffries *et al.* 2006). According to Mellor and Stace-Smith (1977) the common potato viruses in order of increasing difficulty to eradicate are: leaf roll (PLRV), PVA, PVY, aucuba mosaic, PVM, PVX and PVS. Further, PVX and PVS are not only difficult to eliminate by meristem-tip culture are also wide-spread (Morel *et al*. 1968, Mellor and Stace-Smith 1977, Cassells and Long 1982). There is overwhelming evidence that, although PVX and PVS are usually symptomless in potato, they cause significant decreases in yield. Heat treatment of the infected plant prior to meristem culture has been reported to be effective in eliminating these viruses to some extent (Stace-Smith and Mellor 1968, Pennazio 1971, Tapio 1972, MacDonald 1973). A synthetic riboside, ribavirin (1-B-D-ribofuranosvl-1,2,4,-triazole-3-carboxamide) also known as Virazole (Sidwell *et al.* 1972) has been reported to have antiviral activity against several plant viruses (Lerch 1977, Shepard 1977). Cassels and Long (1982) reported that incorporation of ribavirin into tissue culture media of the infected plant prior to meristem culture resulted in higher percentage of progeny plants free of viruses PVX, PVY, PVS and PVM, singly or as complexes. Most of these studies, however, were conducted with a few genotypes.

At the Central Potato Research Institute, Shimla (India) more than 1700 cultivars/ parental lines of potatoes are being maintained in in-vitro genebank (CPRI 2010). Since the source material for in-vitro maintenance came from field genebank, many accessions were found to be positive to PVX and PVS. In the present study, an attempt was made to develop a protocol for elimination of PVX and PVS from infected genotypes. Meristem tip culture in combination with chemotherapy, thermotherapy and chemo-cum-thermotherapy was undertaken on a wide range of genotypes infected with PVX and PVS. In studies on virus elimination it is important to be sure that virus has actually been eliminated. Most of the previous studies used serological tests/ELISA or indicator plants to test the freedom from viruses. In the present study a highly sensitive immuno electron microcopy protocol was used. The efforts led to the development of a protocol for virus elimination, which is more efficient in terms of time and success than those reported previously (Morel *et al.* 1968, Pennazio 1971, Tapio 1972, MacDonald 1973, Mellor and Stace-Smith 1977, Quak 1977, Cassells and Long 1982). This protocol can also be tested in other vegetatively propagated plant species where viruses are serious problems in production of healthy propagules.

MATERIALS AND METHODS

The study was conducted over a period of 3 years (2007-2009) at the Central Potato Research Institute, Shimla and consisted of two experimetns. In the first experiment (Table 1), PVX and PVS infected in-vitro plants of three cultivars K-22 (an old potato hybrid in cultivation in some parts of India), Gulmarg Special (an indigenous old variety of India) and Kufri Khyati (an improved recently released variety) were subjected to chemotherapy, thermotherapy and chemo-cum-thermotherapy before isolating meristems. Based on the results of first experiment, in second experiment a large number of genotypes (Tables 2 and 3) were subjected to thermotherapy or chemo-cum-thermotherapy before isolating the meristems.

In both the experiments methods of culturing and testing were same as described below.

Source material

The plant material of various genotypes used in this study was from the in-vitro genebank maintained at the Central Potato Research Institute, Shimla. These plantlets had been established from nodal cuttings taken from field- or glasshouse-grown plants and cultured under aseptic conditions on MS basal medium (Murashige and Skoog 1962) with vitamins (0.1 mg/l thiamine hydrochloride, 0.5 mg/l pyridoxine hydrochloride, 0.5 mg/l nicotinic acid and 2.0 mg/l glycine) and 3% sucrose, solidified with 0.7% agar. The pH of the medium was adjusted to 5.7 before addition of agar and autoclaving. The general method of culturing and incubation as described by Gopal *et al.* (2008) was followed in establishing the in-vitro cultures. These plantlets were sub-cultured 3-4 times using nodal cuttings so as to produce enough in-vitro plantlets of each genotype for various treatments.

Chemotherapy

Apical nodal cuttings from in-vitro propagated plantlets were sub-cultured on MS medium described above but supplemented with 20 mg/l of ribavirin (Sigma, USA). Ribavirin is thermolabile and was thus filter sterilized, and then added to the autoclaved medium. Plantlets were incubated at 24±1°C and 16 h photoperiod of light intensity 50 micomol $m^{-2}s^{-1}$ for 5 weeks.

Thermotherapy

Three weeks old in-vitro plantlets propagated on normal MS medium described above were incubated at 37±1°C and 16 h photoperiod of light intensity 50 micromol $m^{-2}s^{-1}$ for 2 weeks.

Chemo-cum-thermotherapy

In-vitro plantlets subjected to chemotherapy for 3 weeks as described above were incubated at 37±1°C and 16 h photoperiod of light intensity 50 micromol $m^{-2}s^{-1}$ for 2 weeks. Thus in this treatment also plantlets were grown on ribavirin supplemented medium for 5 weeks (3 weeks at 24±1°C + 2 weeks at 37±1°C).

Meristem-tip culture

Apical shoot tips were excised from the plantlets and the outer leaves and leaf primordial were removed, with the aid of the dissecting microscope, on a laminar airflow cabinet. The apical dome with first pair of leaf primordia, approx. 0.20-0.25 mm in length (depending on the genotype used) were cultured on meristem medium (normal MS medium with vitamins + 0.05 ppm GA_3, 3% sucrose and solidified using 0.3% gelrite). After the meristems turned green (1-2 months depending on meristem tip size and genotype), the medium was changed 3-4 times every fortnight. Once the roots were established, plantlets were sub-cultured on hormone-free MS medium (i.e. without GA_3) with vitamins, 3% sucrose and solidified with 0.3% gelrite.

Immuno electron microscopy

Three weeks old mericlones (Figure 1, obtained after eight subcultures) were used to confirm their freedom from PVX and PVS. Two middle leaflets from the microplant were excised under aseptic conditions and were macerated in 200 µl of 0.1M Sorenson phosphate buffer pH 7.2 and the macerate served as the virus extract. Immuno electron microscopic detection was carried out as per the procedure of Garg and Khurana (1993) which is briefly described here. Electron microscopic grids covered with collodion film were coated with antiserum to the target virus by floating them, film side down, on the micro drops of suitably diluted antiserum for 2-3 minutes followed by washing with distilled water and draining by touching edges on filter paper. The antiserum coated grids were immediately floated on microdrops of the virus extract placed in a humid chamber made of Petridish. They were then incubated at 37°C for 1 hr followed by washing with distilled water, draining and then staining with aqueous uranyl acetate (trapping) or again floating on microdrops of antiserum in the humid chamber and further incubation for another 1 hr followed by washing, draining and staining as above (decoration). Trapping was used to detect the target virus while decoration was employed to confirm the identity of the virus.

RESULTS AND DISCUSSION

The results presented in Table 1 showed that out of 120 plantlets of 3 cultivars exposed to chemotherapy for 5 weeks as many as 103 (85.83%) survived, whereas 2 weeks thermotherapy of 120 plantlets led to survival of only 37 (30.83%) plantlets. Chemo-cum-thermotherapy led to survival of 77.67% (80 out of 103) plantlets. The lowest survival rate was in Kufri Khyati and the maximum was in K-22 in all treatments, and the difference in survival among genotypes was more distinct when plantlets were exposed to thermotherapy.

Table 1 Effectiveness of chemotherapy, thermotherapy and both in elimination of PVX and PVS through meristem-tip culture

Treatment	Genotype	Number of plants		Number of mericlones	
		Exposed	Survived	Tested	Virus-free
Chemotherapy	K-22	40	36	5	0
	Gulmarg Special	40	34	7	0
	Kufri Khyati	40	33	8	0
	Total	120	103	20	0
Thermotherapy	K-22	40	15	3	0
	Gulmarg Special	40	12	8	2
	Kufri Khyati	40	10	2	2
	Total	120	37	13	4
Chemo + Thermotherapy	K-22	36	28	3	3
	Gulmarg Special	34	27	2	1
	Kufri Khyati	33	25	8	8
	Total	103	80	13	12

Meristem-tip culture following chemotherapy for 5 weeks was ineffective in eradicating PVX and PVS from the infected plantlets as none of 20 mericlones of three cultivars could be freed of either PYX or PVS (Table 1). On the other hand chemo-cum-thermotherapy was very effective as 12 of the 13 mericlones were free of both PVX and PVS. Thermotherapy alone was effective in freeing only 4 of the 13 mericlones from both PVX and PVS. Cultivar differed for their response to meristem-tip culture following thermotherapy or chemo-cum-thermotherapy. Kufri Khyati was most responsive to thermotherapy and K-22 was the least (Table 1). Chemo-cum- thermotherapy could free all mericlones of K-22 (3/3) and Kufri Khyati (8/8) of PVX and PVS, whereas out of 2 merilclones of Gulmarg Special only one was free of PVX and PVS.

The second experiment was undertaken to confirm the response of a wide range of genotypes to thermotherapy and chemo-cum-thermotherapy, the treatments found to be effective in experiment 1. Meristem-tip culture of infected plants exposed to thermotherapy alone could free only 21 mericlones of 13 genotypes (Table 2). Remaining accessions were still having PVX or PVS or both. Whereas the meristem-tip culture following chemo-cum-thermotherapy could free 91 mericlones out of 106 tested and was effective in freeing at least one mericlone of all 48 genotypes of both PVX and PVS. (Table 3).

Table 2 Effectiveness of thermotherapy in elimination of PVX and PVS in a wide range of genotypes through meristem-tip culture

Accession	Genotype/Variety	Source	Mericlones	
			Tested	Virus-free
KDW-5	Dihaploid of Kufri Dewa	India	2	2
KLK-13	Dihaploid of Kufri Lauvkar	India	2	2
CP1366	B 721-35	USA	7	1
CP1373	B35-7	USA	7	
CP1409	Fruhmolle	Holland	7	0
CP1411	Gloria	Holland	7	0
CP1412	Herkol	Holland	7	1
CP1425	Prefect	Holland	7	0
CP1433	Sientje	Holland	7	1
CP1491	3053-18	Mexico	7	0
CP1730	Ia1111-8	USA	5	0
CP2141	Kufri Chandramukhi	India	7	0
CP2334	AL575	Peru	2	2
CP2345	MS35.22R	Peru	2	1
CP3417	BW-7	Peru	2	2
CP3419	XY-16	Peru	2	2
CP3544	FGT-8	Peru	2	2
CP3554	P230	Peru	2	2
CP3613	MEX750847	Mexico	2	2
CP3872	MP92-56	India	2	1
Total			88	21

Table 3 Effectiveness of chemo-cum-thermotherapy in elimination of PVX and PVS in a wide range of genotypes through meristem-tip culture

Accession	Genotype/variety	Source	Mericlones	
			Tested	Virus-free
CP1140	Unknown	Unknown	2	2
CP1143	Unknown	Unknown	2	1
CP1159	Unknown	Unknown	2	2
CP1160	Unknown	Unknown	2	2
CP1162	Unknown	Unknown	2	2
CP1175	Unknown	Unknown	2	2
CP1177	Unknown	Unknown	2	2
CP1180	Unknown	Unknown	4	2
CP1187	Unknown	Unknown	4	2
CP1197	Unknown	Unknown	4	2
CP1198	Unknown	Unknown	2	2
CP1207	Unknown	Unknown	2	2
CP1214	Unknown	Unknown	2	2
CP1215	Unknown	Unknown	2	2
CP1218	Unknown	Unknown	2	2
CP1232	Unknown	Unknown	2	1
CP1239	Unknown	Unknown	2	2
CP1243	Unknown	Unknown	2	2
CP1263	Unknown	Unknown	2	2
CP1302	Unknown	Unknown	2	2
CP1373	B35-7	USA	2	2
CP1409	Fruhmolle	Holland	2	2
CP1411	Gloria	Holland	2	2
CP1425	Prefect	Holland	2	2
CP1491	3053-18	Mixico	2	2
CP1534	Aquila	UK	2	2
CP1730	Ia1111-8	USA	2	2
CP1846	Chieftain	USA	2	2
CP1848	Emmet	USA	2	1
CP1850	Persica	USA	2	1
CP1851	Reliance	USA	2	2
CP2134	CIP760147.7	Peru	2	1
CP2141	Kufri Chandramukhi	India	2	2
CP2150	Kufri Lauvkar	India	2	2
CP2158	Kufri sindhuri	India	2	1
CP2345	MS35.22R	Peru	2	2
CP3022	CIP702610	Peru	2	1
CP3081	2070 (54)	Germany	4	2
CP3092	Atlantic	USA	2	2
CP3109	Ica Guantiva	Peru	2	2
CP3351	CIP382196.3	Peru	2	2
CP3479	TS-10	Peru	2	2

CP3480	TS-11	Peru	2	2
CP3484	LM-2	Peru	2	2
CP3493	P55.7	Peru	2	2
CP3557	Kisoro	Peru	2	2
CP3538	CIP676064	Peru	2	2
CP4073	Kufri Pushkar	India	4	4
Total			106	91

The results thus clearly showed that chemo-cum-thermotherapy of the infected plants prior to mericloning was the best option for virus elimination as this treatment proved effective in freeing all accessions from PVX and PVS. On the other hand chemotherapy alone was completely ineffective and thermotherapy was effective in cleaning only15 genotypes out of 22 mericloned. These findings differ from those of Cassels and Long (1982), Klein and Livingston (1982) and Sanchez *et al.* (1991) who had reported that chemotherapy was effective in virus elimination including PVX and PVS from some potato varieties. Cassels and Long (1982) had cultured isolated meristems rather than the infected plants on ribavirin supplemented medium and reported that the continuous presence of ribavirin in the culture medium was required for virus elimination. But for how long the cultures were grown on ribavirin medium was not reported. Wambugu *et al.* (1985) reported that ribiavirin was not effective in virus eradication when shoot-tips were placed on solid media probably due to inability of the chemical to diffuse rapidly enough into the developing tissues. They thus suggested that ribavirin be used in liquid media. This observation, however, could not be supported by the present study in which chemo-cum-thermotherapy was found to be highly effective although ribavirin was used in the solid media.

Fig 1 Mericlone stage used for virus-freedom test

Effectiveness of thermotherapy in eliminating PVX and PVS from only a few genotypes as observed in the present study had also been reported by Mellor and Stace-Smith (1970) and Lozoya-Saldaña and Dawson (1982). The variation in cultivars response to virus elimination as observed in the present study particularly upon therrmotherpay has also been reported by other workers (Lozoya-Saldaña and Dawson 1982, Lozoya-Saldaña and Merlin-Lara 1984, Sanchez *et al*. 1991).

Ribavirin is known to be phytotoxic. Wambugu *et al.* (1985) reported that axillary bud tips treated with ribavirn at 20 mg/l had a survival rate of only 30-40%. However, in the present study a survival rate of more than 85% was achieved by using apical shoot tips (4-5 mm). Sanchez *et al.* 1991) had also suggested that pytotoxic effect may be lower with bigger explant size. By using approximately 0.5-0.7 cm long nodal cutting as explant, they found no adverse effect on survival of the plantlets during 30 days treatment with ribavirn, but root malformation with nodule like structures was observed. Thermotherapy is also known to adversely affect the survival of the treated plants (Lozoya-Saldaña and Merlin-Lara 1984, Sanchez *et al.* 1991). In the present study, the survival rate was only 30.83% with thermotherapy and this improved to 77.67% when chemotherapy was combined with thermotherapy. Perhaps ribavirin alters the physiology of the plantlets such that they are more tolerant of heat-induced stress. Thus chemo-cum-thermotherapy protocols as followed in the present study could be effectively used for assured elimination of PVX and PVS from a wide range of genotypes with a reasonably high rate of survival of the plantlets for isolating meristem-tips. This simple protocol of raising the plantlets on basic MS medium supplemented with 20mg/ribavirn for 3 weeks and incubating them for another two weeks at $37\pm1^{\circ}$C followed by mericloning is now routinely used at the Central Potato Research Institute, Shimla. The plants made free of PVX and PVS by this protocol are expected to be free of other viruses also as those are much easier to remove (Houten etal. 1968, Mellor and Stace-Smith 1977). Houten *et al.* (1968) reported that all of 500 plants obtained from meristem and one leaf primordium were free of PLRV, 70-80% was free of PVA and PVY, but not more than 10% were free from PVX. Further, they reported that of many plants grown from meristem, only two had lost PVX but all were still infected with PVS. Some germplasm accessions used in the present study were also positive to PVA, PVM and PVY. These were simultaneously found to be free of these viruses also upon mericloning following chemo-cum-thermotherapy done for elimination of PVX and PVS. The present protocols (Figure 2) perhaps can also be effective in other vegetatively propagated plant species in cleaning the infected clones from the viruses, and thus may be tried.

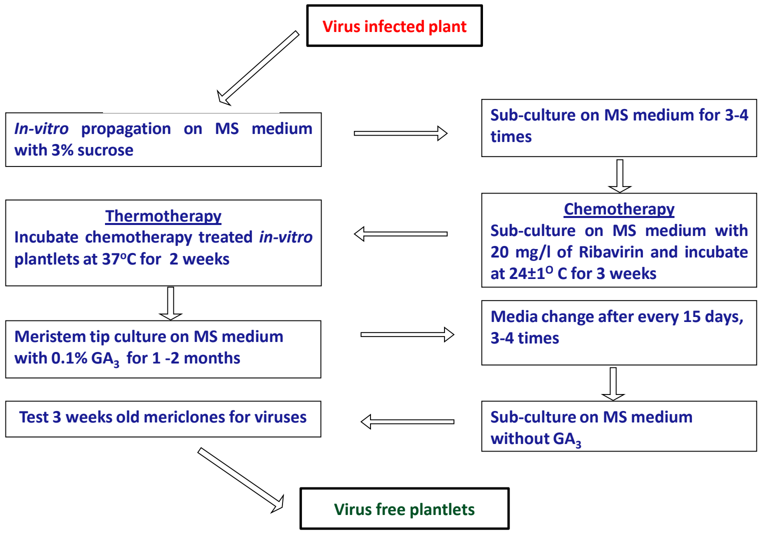

Fig 2 Protocol developed for assured elimination of viruses form potato clones

In conclusion, meristem-tip culture of in-vitro plants subjected to chemotherapy (on MS medium with 20 mg/l of ribavirin) for 3 weeks followed by thermotherapy (at 37±1˚C) for 2 weeks can be effectively used for elimination of two most difficult viruses PVX and PVS in potato.

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