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Ribavirin, Electric Current, and Shoot-tip Culture to Eliminate Several Potato Viruses

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Abstract

To eradicate several viruses such as PVX, PVY, and PLRV which often cause considerable damages to the growth and yields of potatoes, several stems including shoot tips were excised from the potato plants grown for 50 days and electric shock was treated. Shoot tips excised from electric-shocked stems were transferred into the medium supplemented with antiviral compound, ribavirin to examine the combinatorial effect. When treated only with 20 mg/L ribavirin, PVX concentration in the regenerated plantlets was slowly decreased as repeating sub-culture and finally, it took 32 weeks to reach completely PVX-free stock. With an electric shock treatment (10 mA electric current), all the replicates became free from PVY. However, PLRV was not completely eradicated from 94P70-4 and 93P29-3 lines even by treating with 10 mA electric shock. In this case, both electric shock and antiviral compound treatments in axillary buds from the stem segment were successful in eradicating viral contamination.

Key words: Potato, virus, ribavirin, electrotherapy, shoot tip culture, chemotherapy

Introduction

Maintenance of important potato (*S. tuberosum*) genotypes in sterile culture is now a widespread practice as a resource of seedstocks for commercial cultivars and experimental breeding clones (Farrell et al. 1982; Klein and Livingston 1982; Kwiatkowski et al. 1985; Faccioli and Colombarini 1996). Greenhouse and field-grown clones are often infected with viruses and the infection of viruses hinders the expression and evaluation of true performance of genotype.

Meristem culture has been well known as a valuable mean for the production of virus-free stocks (Huang and Murashige 1976) since meristem is an active growing point of the plant shoot and small region composed of rapidly dividing cells. 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 (Lizarrago et al. 1991). Thermotherapy and meristem culture are commonly used for the production of virus-free stocks of potatoes (Pennazio et al. 1976). However, it is time-consuming, requiring several days to a few weeks for completion, and its efficiency on virus eradication is low, from 25% to 40% (Pennazio et al. 1976; Lozoya and Dawson 1982; Lozoya and Merlin-Lara 1984).

One of the antiviral chemicals, a synthetic riboside, ribavirin was known to be used as supplemental agents during the culture of excised shoot tip for the elimination of three of the major potato viruses, PVX, PVY, and PVS (Klein and Livingston 1982; Wambugu et al. 1985). Furthermore, ribavirin has been identified to be the most effective viricide for the plant-virus systems (Lizarrago et al. 1991). The majority of viruses could be eliminated from potato plantlets without the need for meristem culture that sufficient stringency was applied through therapy procedures with ribavirin (Griffiths and Jack 1988).

Recently, electric pulses are reported as stimulants on plant differentiation *in vitro* (Lozoya and Madrigal-Vargas 1985) and electrical properties of plant tissue are considered in a wide range of physiological studies (Goldworthy 1987). However, the effect of electric current to eliminate viruses from plant tissue has received little attention (Quacquarelli et al. 1980).

In this preliminary trial, several antiviral chemicals were applied for elimination of major potato viruses, PVX, PVY and

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PLRV. In these antiviral chemicals, ribavirin was the most effective to eliminate viruses. In this study, it is proposed that electric shock treatment prior to the treatment of antiviral chemicals, ribavirin may enhance the regeneration rate of virus-free plantlets.

Materials and Methods

Plant materials and tissue culture

Five different potato lines (94P70-4, 93P29-3, 93P42-1, 89L92-3, 89L92-4) obtained from yield trial test at National Alpine Agricultural Experiment Station, RDA and one wildtype 'Chuncheonjaerae' originated from Chuncheon, Gangwon province, South Korea were used. To investigate the degree of eradication of viruses, the wildtype potato line was compared with cultivated potato lines.

Potato plants were grown in a greenhouse for 50 days. For plant propagation, tissue culture media, SIM (shoot inducing medium), SMM (shoot multiplication medium), and RM (ribavirin medium) were used as indicated in Table 1. Axillary buds were removed from stem segment and sterilized by shaking them in 1% aqueous NaOCl solution with several drops of Tween-20 for 15 min. Growing tips, 0.5-1.0 mm long, were excised from each bud and cultured by shaking in the shoot-tip culture medium consisting of basic MS salts, 0.2 mg/L GA_3, 0.04 mg/L kinetin, 0.1 mg/L IAA and 30 g/L sucrose (Table 1). In every eight weeks, sub-culture was carried out and incubation was done at 35 micro Einstein (μE), in a 16 h/day light regime, at 23±1°C.

Treatments for virus elimination

All the samples used in this study were infected severely with PVX, PVY, and PLRV. The most vigorous stems were selected, and the stem segments with approximately six axillary buds were treated with one of the followings (Figure 1):

Table 1. Components of media used in this study

	Medium		
Components (mg/L)	SIM[a]	SMM[b]	RM[c]
IAA	0.1		
GA_3	0.2	0.1	
Kinetin	0.04		
Ribavirin			20.0
MS mix (g/L)	4.4	10.4	4.4
Sucrose (g/L)	30.0	30.0	30.0
Agar (g/L)	8.0		8.0

[a]Shoot inducing medium.
[b]Shoot multiplication medium.
[c]Ribavirin medium.

electric currency of 5, 7, 10 mA for five minutes. Electricity was supplied by an electrophoresis power (LKB BROMMA 2297 Macrodrive 5). Electric-treated axillary buds were removed from stem segment and shoot tips were excised after sterilizing with sodium hypochlorite and cultured by shaking in SIM with or without 20 mg/L ribavirin. To analyze the separate effects of ribavirin on the eliminating viruses, the excised shoot-tips with no electric shock were also cultivated in the SIM supplemented with 20 mg/L ribavirin. In every cases, sub-culture were carried out in every eight weeks.

Virus detection

Detection of viruses was performed at the end of each 8-week of cultures with ELISA-DAS (Agdia company, USA) according to Clark and Adams (1977). Shoots (0.25-0.50 g) were homogenized in 1.0 mL of extraction buffer to give a constant ratio of 1:4. Tissue samples from healthy and infected mother plants growing in the greenhouse were used as negative and positive controls. Precoated ELISA plates (96-well polystyrene) containing 200 μL of the sample/conjugate macerate per assay were incubated for 18 hr at 4°C. After washing, they were incubated at 23°C with 200 μL of p-nitrophenol substrate added to each well. After 1-2 hr, the optical density at 405 nm was measured using ELISA reader (Tecan, Spectra-Rainbow, Austria).

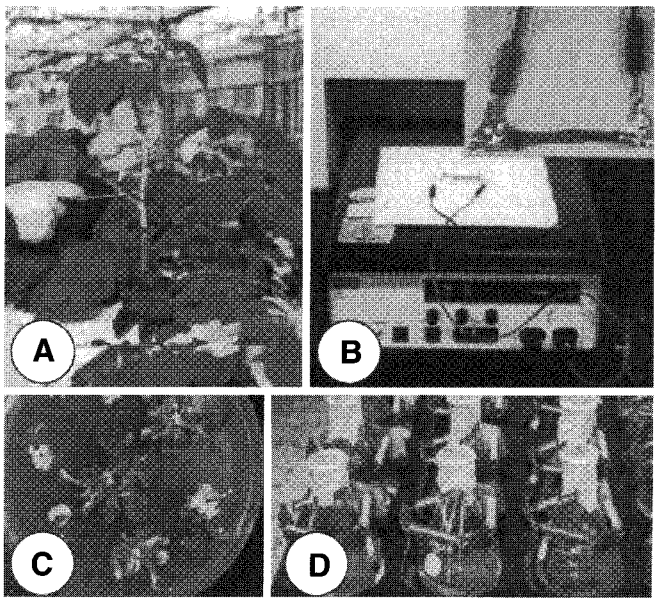

Figure 1. Procedures of electrotherapy and plantlet regeneration *in vitro*. A: cutting the stem of 50-day-old greenhouse grown potato plants infected with PVX, PVY and PLRV, B: the stem segment treated with current intensity, C: induction of shoots from shoot- tips treated with current intensity, and D: multiplication of shoots free from viruses.

Results and Discussion

Plant regeneration

To investigate the effect of electric shock and chemical treatments on the regeneration rate of plantlet during the tissue culture, initially the regeneration and growing pattern of each regenerated plants were observed. As indicated in Table 2, the rate of regeneration and growth of shoots were not greatly retarded by treatment of ribavirin (20 mg/L) supplemented in the culture media while electrical shock treatment resulted in a serious reduction in the regeneration rate. But, according to Wambugu et al. (1985), ribavirin treatement reduced growth rate in culture and bud tip cultures treated with 20 mg/L ribavirin showed severe growth abnormalities which included chlorosis, stunting, root inhibition and base callus formation. Depending on the genotypes, the effect of the electric shock treatment on the regeneration rate was quite different, 21.3% to 91.7% (Table 2), while non-treated control samples resulted in 86.5% to 96.3% regeneration rate. Electric current also seemed to affect organogenesis and growth *in vitro*.

Chemotherapy

To investigate the effect of antiviral chemicals, ribavirin was treated during the course of sub-culture and the concentration of virus was measured with DAS-ELISA test. The 20 mg/L ribavirin therapy was most effective in eliminating PVX from all shoots of the six clones in the yield trial test. As shown in Table 3, at the end of the forth sub-culture (32 weeks), more than 99% replicates were free from PVX. As a result of these data, ribavirin treatment, by itself, was more than enough to completely eradicate PVX. It was also reported that three viruses, PVM, PVS, and PVX could be eliminated by ribavirin treatment without any other means such as the use of meristem culture (Helene et al. 1989). However, it was indicated that some species, such as PVY and PLRV were not completely eradicated by the treatment of ribavirin alone and suggested that electrotherapy or combinatorial treatment with other means might be necessary.

Electrotherapy

According to several previous reports, exposures to mild electric current, 5 to 10 mA for 5 min, prior to *in vitro* culture, may improve regeneration of plant tissue (Goldsworthy 1987; Lozoya et al. 1996). In addition, electrical properties of plant tissue are considered in a wide range of physiological studies (Nelson 1973). At the end of the forth sub-culture (32 weeks), most replicates exposed to 7 mA were confirmed as PVY-free except for 89L92-4 which become PVY-free through the exposure to 10 mA (Table 4). A preliminary result of this study clearly indicated that electricity treatment seemed to affect the elimination of virus effectively. As indicated in Figure 2, the concentration of PVY was very low by the treatment of electricity but another virus, PLRV was not completely eradicated in 94P70-4 and 93P29-3 lines, even though it was exposed to 10 mA.

Combination of chemotherapy and electrotherapy

For a particular virus, PLRV in 94P70-4 and 93P29-3 clones, it was assumed that combinatorial treatment with electricity as well as ribavirin might be effective in the eradication. To eradicate PLRV from the lines used in this study, some axillary buds from the segment treated by electricity were excised and cultured in the medium with ribavirin (20 mg/L). Every eight-weeks,

Table 2. Effects of electric and ribavirin treatments in potato shoot-tip culture on the rate of plant regeneration (32 weeks after continuous sub-culture)

	Rate of regeneration (%)						
Lines	Control	5mA	5mA+R[a]	7mA	7mA+R	10mA	10mA+R
94P70-4	92.2 a[c]	46.6 cd	42.3 d	56.3 b	53.3 bc	23.2 e	23.7 e
93P29-3	86.5 a	45.8 d	36.0 e	54.2 c	62.5 b	21.3 f	20.5 f
93P42-1	96.0 a	88.5 b	84.0 b	45.8 c	40.0 d	45.8 c	28.0 e
89L92-3	96.3 a	91.7 a	82.6 b	43.5 c	37.5 d	28.0 e	21.7 f
89L92-4	94.1 a	33.3 c	52.0 c	60.0 b	54.2 bc	52.0 c	44.0 d
Wildtype[b]	88.7 a	43.7 bc	45.1 b	39.7 bc	42.1 bc	37.3 c	36.9 c
Mean	92.3 a	58.3 b	57.0 bc	50.0 cd	48.3 d	34.6 e	29.1 e

[a]Adding 20 mg/L ribavirin in the medium.
[b]Originated from Chuncheon, South Korea.
[c]Mean separation within columns by Duncans multiple range test, at 5% level.

Table 3. Separate effects of continuous sub-cultures with ribavirin on eradication of PVX in potato shoot-tip culture

	Regenerated PVX-free plants (%)						
Lines	Control	RF[a]	8-wks[b]	16-wks	24-wks	32-wks	40-wks
94P70-4	1.1 d[d]	54.0 c	54.1 c	58.2 c	65.0 b	99.4 a	100.0 a
93P29-3	2.0 e	56.1 d	57.2 cd	61.6 c	77.3 b	100.0 a	100.0 a
93P42-1	2.1 e	62.3 d	67.0 d	76.1 c	82.1 b	98.1 a	98.5 a
89L92-3	0.0 d	43.2 c	43.2 c	44.3 c	50.7 b	100.0 a	100.0 a
89L92-4	1.2 e	50.2 d	51.6 d	61.1 c	72.1 b	99.1a	100.0 a
Wildtype[c]	2.4 d	57.6 c	58.0 c	62.0 c	84.4 b	100.0a	100.0 a
Mean	1.5 d	53.9 c	55.2 c	60.6 c	71.9 b	99.4a	99.8 a

[a]Cultured in ribavirin-free medium.
[b]Weeks.
[c]Originated from Chuncheon, South Korea.
[d]Mean separation within columns by Duncans multiple range test, at 5% level.

Table 4. Effects of electric current and ribavirin on eradication of PVY in potato shoot-tip culture (32 weeks after continuous sub-culture)

	Regenerated PVY-free plants (%)						
Lines	Control	5mA	5mA+ R[a]	7mA	7mA+R	10mA	10mA+R
94P70-4	1.1 d[c]	42.1 c	59.4 b	100.0 a	100.0 a	100.0 a	100.0 a
93P29-3	2.0 e	51.0 c	67.1 b	99.5 a	99.7 a	99.9 a	100.0 a
93P42-1	2.1 e	58.5 c	69.0 b	99.1 a	99.5 a	99.8 a	99.5 a
89L92-3	0.0 d	49.3 c	68.1 b	99.9 a	100.0 a	100.0 a	100.0 a
89L92-4	1.2 e	41.8 d	51.7 c	61.6 b	59.9 b	99.2 a	99.8 a
Wildtype[b]	2.4 d	53.3 c	64.2 b	99.8 a	99.6 a	99.8 a	99.5 a
Mean	1.5 d	49.3 c	63.3 b	93.3 a	93.1 a	99.8 a	99.8 a

[a]Adding 20 mg/L ribavirin in the medium.
[b]Originated from Chuncheon, South Korea.
[c]Mean separation within columns by Duncans multiple range test, at 5% level.

Table 5. Effects of electric current and ribavirin on eradication of PLRV in potato shoot-tip culture (32 weeks after continuous sub-culture)

	Regenerated PLRV-free plants (%)						
Lines	Control	5mA	5mA+R[a]	7mA	7mA+R	10mA	10mA+R
94P70-4	1.1 e[c]	37.3 d	40.2 cd	45.7 c	68.3 b	71.4 b	100.0 a
93P29-3	2.0 e	39.1 d	38.5 d	48.3 c	69.5 b	68.3 b	100.0 a
93P42-1	2.1 d	51.5 c	53.1 c	68.2 b	73.2 b	95.3 a	99.5 a
89L92-3	0.0 f	48.0 e	47.0 e	57.0 d	75.4 c	94.6 b	99.3 a
89L92-4	1.2 e	53.2 d	55.1 cd	57.8 c	63.6 b	100.0 a	100.0 a
Wildtype[b]	2.4 f	45.2 e	51.1 d	63.1 c	77.1 b	98.1 a	100.0 a
Mean	1.5 f	45.7 e	47.5 e	56.7 d	71.2 c	88.0 b	99.8 a

[a]Adding 20 mg/L ribavirin in the medium.
[b]Originated from Chuncheon, South Korea.
[c]Mean separation within columns by Duncans multiple range test, at 5% level.

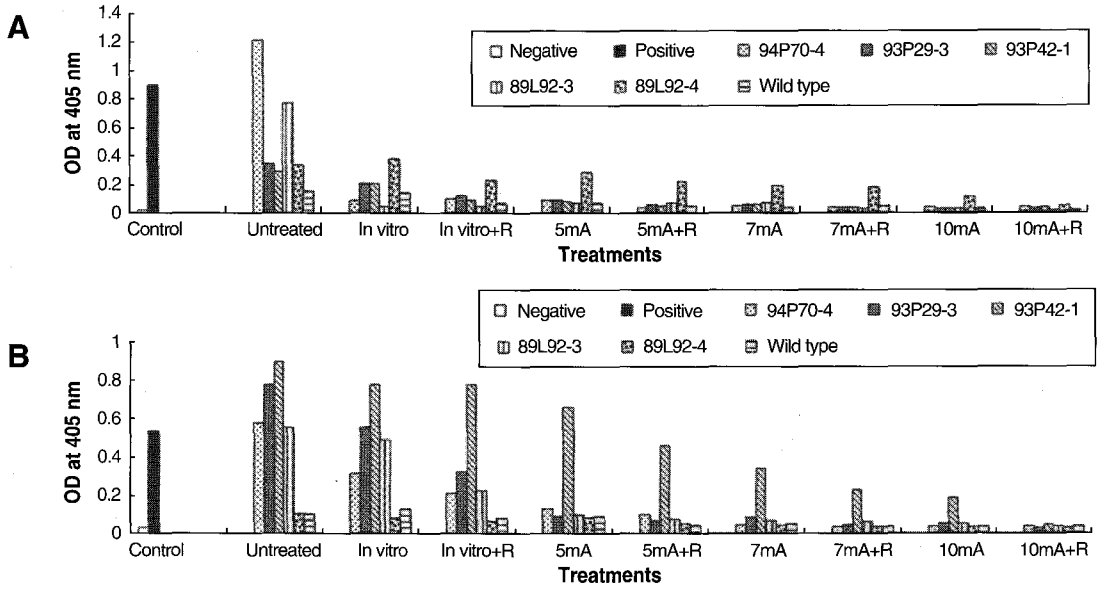

Figure 2. Changes of PVY (A) and PLRV (B) concentrations of shoot extracts after ribavirin and electricity treatment (32 weeks after continuous sub-culture).
*Negative; virus-free plantlets, Positive; infected plantlets by viruses, Wildtype; originated from Chuncheon, South Korea, R; adding 20 mg/L ribavirin in the medium.

the medium was exchanged with new one adding 20 mg/L ribavirin. After 32-week of sub-culture, it was confirmed that PLRV was eradicated in all replicates treated by electric current 10 mA and cultured in the medium with 20 mg/L ribavirin (Table 5). Figure 2 shows that the dramatical reduction of PLRV concentration of the plantlets treated with electricity and ribavirin, simultaneously. The plants freeing from viruses were further propagated *via* nodal cuttings in the medium without ribavirin because plants were propagated better without antiviral agent.

According to these results, efficiency of electrotherapy or ribavirin for eradication of potato viruses was somewhat low, but these methods were found to be technically easier and simpler than meristem culture or heat therapy. Furthermore, regeneration rate was higher than other methods and less time-consuming since shoot tips were applied instead of meristem.

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