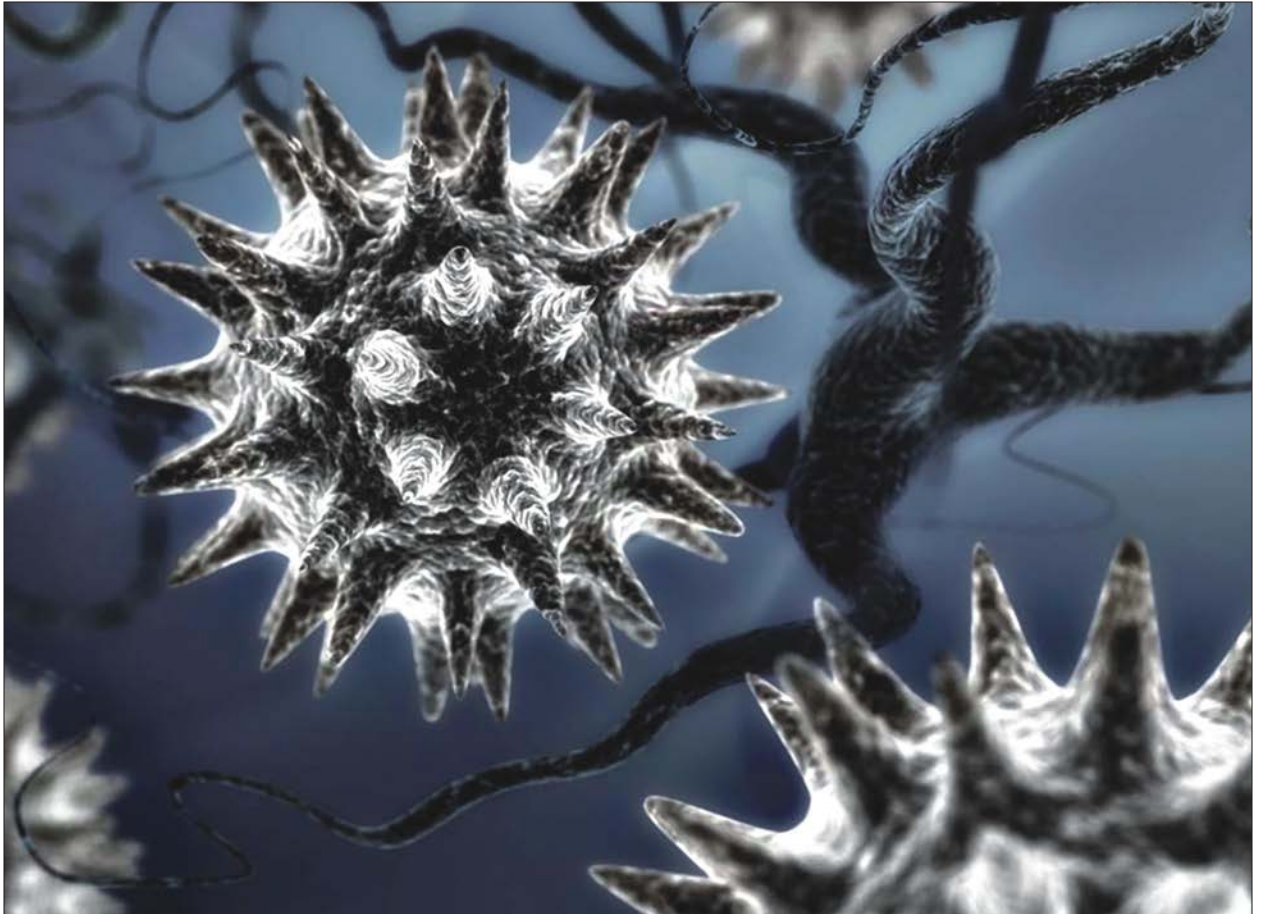




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Evaluation of Some Therapies to Eliminate Potato Y Potyvirus from Potato Plants

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Abstract: Viral infection of potato (*Solanum tuberosum* L.) plants is very important due to their effect on potato yield and degeneration of seed tubers. This study aimed to eliminate Potato Virus Y (PVY) by tissue culture technique using different therapies, such as thermo-, chemo- and electrotherapies. Potato plants cv. Diamond cultivated at Faculty of Agriculture farm, Sohag University were tested by Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) using antisera against PVY, Potato Virus X (PVX) and Potato leaf roll virus (PLRV). The results indicated the occurrence of single and mixed infections of three viruses in potato plants. Survey results indicated highly distribution of PVY infected plants, which its yield was affected strongly. Tubers of potato plants which gave a positive reaction to PVY only were collected at harvest, stored in cold store room for 3 months and then planted in a greenhouse to obtain materials for virus elimination studies. Virus status *in vitro* plantlets was determined by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) and biologically test before and after treatments. The two cycles of thermotherapy at approximately 37°C, during 40 and 30 days, resulted in 33.3 and 14.2% PVY elimination, respectively. Thus, these results indicated that thermotherapy is moderately efficient for viral eradication. Chemotherapy was undertaken with 20 mg L⁻¹ ribavirin (RBV); it showed the medium rate of virus elimination (30.0 - 42.9% virus-free plantlets) when added to the tissue culture media. Several stems including axillary buds were excised from the potato plants grown for 45 days and electric-shocked treated. Stems were directly connected to the electrodes of power supply or indirectly by immersion of plant tissue in electrified water in a wide range of intensity-time. Axillary buds excised from electric-shocked stems or tissues immersed in electrified water, were transferred into the medium supplemented with or without ribavirin (RBV) to examine its combination effect. With an electric shock treatments alone (5, 10 and 15 mA electric current), the replicates become PVY-free in rates 53.8, 72.7 and 87.5%, respectively. Simultaneous, RBV and electric shock had higher efficiency for PVY elimination, reaching rates of healthy plantlets of 66.7% with 5 mA, 90.0% with 10 mA and 100.0% with 15 mA. In this case, both electric shock and antiviral compound treatments in axillary buds from stem segment were successful in PVY-elimination; hence this treatment reported the most consistent. The resulted virus-free plantlets were further propagated via., nodal cutting in the medium without antiviral agent and then used as a mother plants for minituber production. Total yield was significantly higher in plants grown from virus-free seed minitubers when evaluated under field conditions in comparison with those grown from viral-infected tubers.

Key words: *Solanum tuberosum*, virus-free, thermotherapy, chemotherapy, electrotherapy, PVY, ELISA, elimination

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most widely distributed crop in tropical and subtropical zones of the world and provide a stable food for millions of people. Potato production is threatened by different biotic agents such as bacteria, fungi and viruses. Viral infected plants characterized by a decrease in vigor, productivity and resistance to other pathogens (Sangar *et al.*, 1988). The occurrence of potato viruses, including Potato Virus Y (PVY) was observed in commercial fields (Nascimento *et al.*, 2003; Biswas *et al.*, 2005), developing in a single and mixed infections. High virulence of these viruses, continuous introductions of the viruses through imported seeds and recurrent occurrence of the carrier/vector of some these viruses are the main impediments in directly controlling viral infections (Ahmad and Ahmad, 1995). Potato Virus Y (PVY) is the type species of the genus *Potyvirus*, in the family *Potyviridae* (Kitajima *et al.*, 1997). Symptoms induced by PVY vary from an almost imperceptible mosaic up to severe necroses and premature death of plants, depending on cultivar and viral strain (Barker, 1994; Sturz *et al.*, 2000; Robert *et al.*, 2000). Yield decrease resulting from PVY was found to range from 14 - 88% (Spaar and Kleinhempel, 1986), while De Bokx and Huttinga (1981) pointed out that PVY reduced yield by 10-80% depending on the virus strain, potato cultivar and inoculation time. Hane and Hamm (1999) presented that there are decrease in total and marketable yield by using PVY infected potato tubers in the field. Seedborne infected plants had fewer and smaller tubers than did plants grown from virus-free ones. Whitworth *et al.*, (2006) found that PVY reduces yield in many cultivars. Seed tuber quality is an extremely important factor for potato yield, since it is a vegetative-propagated plant and viral disease agents are easily transmitted through the tubers (Sturz *et al.*, 2000; Nascimento *et al.*, 2003). One of the most viable methods for obtaining virus-free stocks from propagative materials that comes from infected plants is viral eradication by using tissue culture techniques, aided or not by thermo-and/or chemotherapies (Mellor and Stace-Smith, 1970; Awan *et al.*, 2007). These methodologies allow quick propagation of plant materials, producing healthy plants in a short period of time. Thermotherapy and meristem culture are commonly used for the production of virus-free stocks of potatoes (Faccioli and Rubies-Autonell, 1982; Faccioli and Colombarini, 1996). Klein and Livingston (1983) described that, Potato Virus X (PVX) and Potato Virus Y (PVY) were eliminated by meristem tip culture and chemotherapy using ribavirin (1- β -D-ribofuransyl-1,2,4 triazone-3-carboxamide), but the time required for regeneration of the tips was longer than the untreated controls. Cassels and Long (1982) and Cassels (1987), reported that the inclusion of ribavirin in tissue culture media resulted in a high frequency of elimination of the potato virus complex X, Y, S and M. The application of electric pulses to eliminate viruses from plant tissue has recently received much attention. Using an electrotherapy Quacquerelli (1980) obtained symptomless almond plants. Also, by using electrotherapy apparatus developed in Cuba (Patent Cuba 37/95 AO 1C/08 1524/97), treated garlic (*Allium sativum* L.), sugar cane (*Saccharum* sp.), banana (*Musa* sp.) and araceas (*Xanthosomas* and *Colocasia*) for potyvirus, Luteovirus, Cucumovirus and Carlavirus elimination, respectively (cited from Hernandez *et al.* (1999)). For potato, Yi *et al.* (2003) and Dhital *et al.* (2008), reported PVX, PVY and PLRV elimination in approximately 40% of regenerated plants. This study started because high yielding commercial varieties or lines shown durable resistance against potato viruses in Egypt are not available. So that, the study aimed to evaluate the efficiency of different therapies for PVY elimination in potato plants and find out the best one for eradication of virus and higher plant regeneration. Production of virus-free minitubers from mother plants for using it as potato tuber was also undertaken.

MATERIALS AND METHODS

Serological Detection of Potato Materials

A preliminary test was carried out with randomized sample leaves of 122 potato plants of cv. Diamond that exhibited viral infection symptoms and others showed no symptoms. The samples were obtained from the experiment cultivated on the 9th of October, 2005 in Faculty of Agriculture Farm, Sohag University. The experimental field were prepared and shaped to ridges 70 cm apart. Each experimental plots was 3×3.5 m (1/400 fed.) contained four ridges (70 cm wide and 3.5 m long). The seed tubers of potato cultivar were planted on the top of the ridges in hills at 25 cm apart. The normal culture procedures known for commercial potato production other than the applied treatments were performed. The samples were taken 35 days after planting and then plants covered with Agryl P17 Lutradur® (Lutrasil Spinvil Comp., Liebigstr 2-8, Germany) for insect protection. The samples were indexed by Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) test according to Hobbs *et al.* (1987), using antisera against PVY, PVX and PLRV, each at the concentration of 1:1000 (v/v). The field plants having different types of infection were marked and the tuber yield was determined at harvest.

Obtaining PVY-Infected Potato Stock

Tubers from 52 plants that reacted positively to PVY only were collected at harvest. The yield from each plant was placed in individual nylon mesh bags stored in storage room for 3 months and then planted in a greenhouse to obtain materials for further studies. After sprouting, plantlets were indexed by using PVY-antiserum to confirm the occurrence of single infection by PVY in the selected material. To be sure of freeing the plants from some other viruses, growing plants were tested biologically using specific indicator plants for each virus. Some leaves from each plant were grinding in 0.01 M pH 7.0 phosphate buffer and inoculating the following differential hosts: *Gomphrena globosa* L. and *Nicotiana glutinosa* (to detect, PVX and *Tobacco ring spot virus*), *Datura metale* and *Solanum tuberosum* (for PVY and potato virus A), *Chenopodium amaranticolor* (for potato virus S and potato mop-top virus) and *Nicotiana tabacum* cvs. White Burley and Samsun (for *Tobacco ring virus* and *Tobacco necrosis virus*). By this method, plenty of infected tissues were available, since tissues were harvested after 45 days then used in subsequent tests.

Potato Cultivation by Tissue Culture

For plant propagation, Shoot Inducing Medium (SIM), Shoot Multiplication Medium (SMM) and Root Inducing Medium (RIM) were used as shown in Table 1, according to Yi *et al.* (2003) and Farzana *et al.* (2007). Electric-treated axillary buds were removed from stem segments and sterilized by shaking them in 6% commercial bleach solution with several drops of Tween 20 for 10 min, followed by 60% ethanol for 5 min. The meristem tips (about 1.0 mm long) were excised from

Table 1: The chemical components of media

Components	Medium type**		
	SIM	SMM	RIM
Indolacetic acid (mg L ⁻¹)	0.1	0.0	0.10
Gibberelic acid (mg L ⁻¹)	0.2	0.2	0.20
Kinetin (mg L ⁻¹)	0.0	0.0	0.04
Ribavirin (mg L ⁻¹)	20.0	20.0	20.00
MS mix* (g L ⁻¹)	4.4	4.4	4.40
Sucrose (g L ⁻¹)	30.0	30.0	30.00
Agar (g L ⁻¹)	7.0	0.0	0.00

**SIM: Shoot Inducing Medium; SMM: Shoot Multiplication Medium; IM: Root Inducing Medium. * MS basic salts according to Murashige and Skoog (1962)

each bud with needles and razor blades, under a low-power microscope placed in sterile laminar-flow, then cultured in SIM at $25\pm 2^{\circ}\text{C}$ for 4-6 weeks. Differentiated shoots were excised then single node cuttings (1.0 cm length) were made according to Kluge *et al.* (1990) and then transferred to SMM for shoot propagation and RIM for root formation. The pH of all media was adjusted at 6.0 by using 1 N NaOH and immediately distributed into containers which were then sealed with aluminum foil and autoclaved at 121°C for 20 min. Containers were incubated in a growth room, under a temperature regime of $25\pm 2^{\circ}\text{C}$ and photoperiod cycle of 16/8 h as light/dark, provided by fluorescent tubes with light intensity of 2500 lux according to Jayasree *et al.* (2001) for potato.

Treatments for Virus Elimination

All the samples used in this study were obtained from PVY-potato infected stock growing under greenhouse conditions for 45 days.

Thermotherapy

Thermotherapy was carried out in two cycles according to Nascimento *et al.* (2003). Firstly, 14 PVY-infected stock plants, showing root growth and leaves, were submitted for 40 days to temperature of $37\pm 2^{\circ}\text{C}$ and then viral indexed. By using single nodal cuttings grown in SMM and RIM maintained at $25\pm 2^{\circ}\text{C}$, several plantlets were subcultured from survived and remained infected plants after first cycle. Single node cutting about 1.0 cm in length having one bud, were taken from the PVY-infected plants growing in greenhouse. These were sterilized as mentioned above. Explants were cultured individually and maintained at $25\pm 2^{\circ}\text{C}$ under 16 h photoperiod (Awan *et al.*, 2007). By using this method, a great number of infected plantlets, according to performed indexing were originated. Thirty five plantlets reached between 8 and 10 cm were utilized in the second cycle. During the second cycle, 35 plantlets established *in vitro* were transferred to growth chamber at $37\pm 2^{\circ}\text{C}$, whereas they remained for 30 days then indexed.

Chemotherapy

To analyze the separate effects of ribavirin on PVY elimination, the excised meristem-tips with no electric shock were also cultivated in the SIM, SMM and RIM supplemented with 20 mg L^{-1} ribavirin. Ribavirin was filter-sterilized and added to culture media.

Electrotherapy

The most vigorous stems were selected and treated with one of the followings: (1) the stem segments with approximately 5 axillary buds were directly connected to the electrodes for electric current intensity-time combinations: 5, 10 or 15 mA for 5 or 10 min, (2) stem segments with approximately 2 axillary buds were washed for 5 min in a detergent bath and then fixed in the electrophoresis chamber containing NaCl solution (1 M) with the same range of intensity-time. Electricity was supplied by electrophoresis power supply (LABCONCO power supply 433-3240) as shown in Fig. 1. After the treatment, the axillary buds were removed, surface-sterilized then meristem-tips were excised and cultured on SIM. For studying the combination effect of electrotherapy and chemotherapy, meristem-tips excised from electric shocked axillary buds were cultured in the medium supplemented with ribavirin (20 mg L^{-1}).

The parameters studied were % survival of plantlets after treatments; % virus elimination which was determined by DAS-ELISA and biologically test and therapy efficiency according to Lozoya-Saldan a *et al.* (1996).

PVY-Detection

Detection of PVY was performed at the end of cultures with Double Antibody Sandwich-Enzyme linked Immunosorbente Assay (DAS-ELISA) according to Clark and Adams (1977).

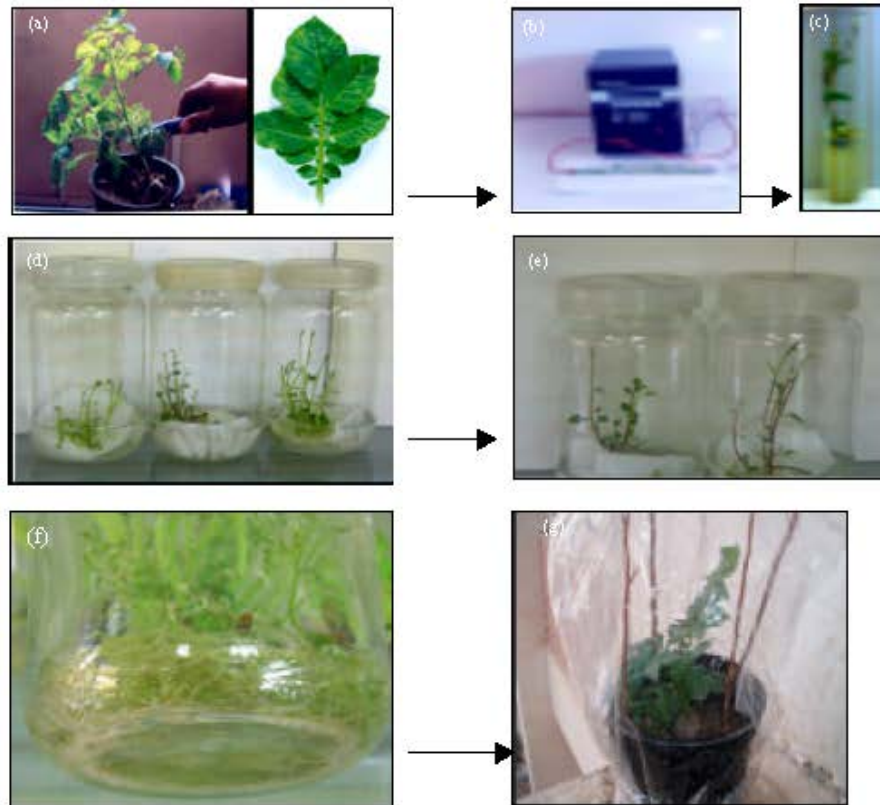


Fig 1: Procedure of electrotherapy and plantlet regeneration *in vitro* (a) Cutting the stem of 45 day-old greenhouse grown potato plants infected with PVY, (b) The stem segment treated with current intensity, (c) Induction of shoots from meristem-tips treated with current intensity, (d) Multiplication of shoots free from virus, (e) multiplication of plantlets, (f) Roots induction and (g) Plantlets acclimatization

Shoots (0.50 g) were homogenized in 1.0 mL of extraction buffer to give a constant of 1:4. Tissue samples from healthy and infected mother or stock plants growing in the greenhouse were used as negative and positive control.

Acclimatization and Minutuber Production

Plantlets submitted to chemotherapy, thermotherapy and electrotherapy, regenerated with roots and a well developed aerial part (about 5-7 leaflets), were removed from the culture medium and their roots were washed-free of media in running water. These plants were acclimated in pots containing a sterilized mixture of soil, vermiculite and organic matter, in the rate of 2:2:1 and kept under a transparent polystyrene cover, for two weeks. The plants remained without incidence of direct sunlight during this period in greenhouse at 22-25°C. In the first weeks of acclimation, plants were irrigated daily with Hoagland nutrient solution (Gold and Faccioli, 1972). After 3 weeks survivor plantlets were indexed serologically and then used as mother plants. The virus-free plantlets can be transplanted at high density in pots filled with previous sterilized mixture in aphid-proof greenhouse to be used as mother plants for minutuber production. Ten plantlets per pot (20 cm) were used.

Virus-Free Minutubers Evaluation

The virus free minutubers, PVY infected potato tubers and PVY, PVX and PLRV mixed infected tubers were planted in open field on 10th of October, 2006 to compare among them. Each treatment was cultivated in individual plot (1/400 fed), which contain three replicates. Five plants from each plot were taken for measuring the vegetative growth parameters; i.e., plant height (cm), number of tubers/plant, average weight of tuber and weight of tubers/plant.

RESULTS AND DISCUSSION

Stock Indexing and *in vitro* Cultivation of PVY-Infected Plants

When the antisera against PVY, PVX and PLRV were utilized in the DAC-ELISA test, 79 and 43 plants, presented positive and negative reactions, respectively. The utilization of specific antisera allowed identification of individual and simultaneous presence in several combinations of the PVY, PVX and PLRV viruses (Table 2). PVY was detected in 62 out of 122 indexed potato plants; of which 52 showed single infection and 10 mixed infections. On the other hand, the total yield was reduced especially on mixed infection plants compared to healthy ones (Table 2). From the tubers bearing from single PVY-infection, 33 PVY-infected plants were obtained in greenhouse; the other one plant was found have mixed infection by PVY and PVX according to biological test. The PVY infected plants were used in thermotherapy (14 plants) and electro- and chemotherapy (19 plants). This study supports the information on the legitimate use of specific antisera to obtain healthy material, just by verifying the presence or absence of viruses. Using the double antibody coating ELISA test for viral detection in potato, Nascimento *et al.* (2003) observed the same sensibility when using individual or mixed antisera. The utilization of specific antisera allowed identification of individual and simultaneous presence, in several combinations, of the PVY, PVX and PLRV viruses in plants (Table 2). On the other hand, biological test confirmed the ELISA results, except one plant which neglected because it reacted positively to PVX using *N. glutinosa*. So that, virologists are often used bioassay to test the presence of viruses because they tend to be fairly sensitive and indication of the presence of infection material.

Thermotherapy of Plants Multiplied *in vitro* by Single Node Cutting

From the 14 infected plants established in greenhouse and submitted to the first thermotherapy cycle (40 days), 6 plants were survived and indexed. The indexing showed that two survivors were virus-free, indicating that 33.3% efficiency was achieved for PVY elimination (Fig. 2). 224 single node cutting was obtained from infected plantlets grown on SIM and then propagated in RMM. From these, 166 plantlets were produced, corresponding to a 74.1% regeneration percentage. When 35 from 166 plantlets were submitted to the second thermotherapy cycle (30 days), only five produced PVY-free progenies, thus achieving a virus elimination index of 14.2% (Fig. 2). This study indicated that,

Table 2: Potato plants indexing by using specific antisera

Viruses detected*	Reaction to antiserum**	No. of plants positively reacted	Yield / plant No. of tubers	Weight of tubers
PVY	+	52	8.0	1.040
PVX	+	4	9.25	1.258
PLRV	+	11	6.5	0.806
PVY + PVX	+	3	5.3	0.634
PVY + PLRV	+	4	5.0	0.575
PVX + PLRV	+	2	5.0	0.555
PVY + PVX + PLRV	+	3	4.6	0.475
Control	+	0	12.4	0.292

*PVY: Potato virus Y; PVX: Potato virus X; PLRV: *Potato leaf roll virus*. ** +: Positive reaction, -: Negative reaction

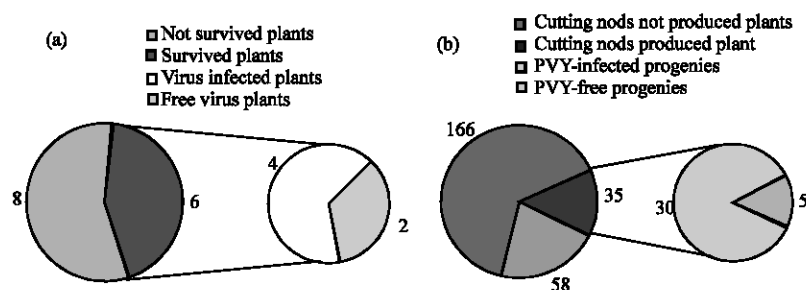


Fig. 2: Effect of thermotherapy on PVY elimination, (a) First cycle, 2 plants from 6 survived plants were PVY-free, but 4 were PVY infected plants and (b) thirty five plantlets resulted from infected cutting nodes taken after first cycle and then submitted to the second thermotherapy

37°C for periods ranging from 30 to 40 days are not efficient for viral eradication. So that, using temperature, can not be considered adequate method for virus elimination in potato plants cultivated *in vitro* from single nodal cuttings. These results are not agreement with that obtained by Faccioli and Rubies-Autonell (1982); they eliminated PVX and PVY from *in vitro* potato plants by using 35°C for periods ranging from 16 to 27 days. On the other hand, Nascimento *et al.* (2003) reported free significant elimination of PVY. The percentage of virus elimination with thermotherapy can be increased by the simultaneous adoption of other techniques (Hollings, 1965; Stace-Smith and Mellor, 1968; Slack, 1980). The effect of heat on viruses is not well understood but it believed to be effective in inhibiting viral replication and synthesis of movement proteins mainly by blocking transcription (Mink *et al.* 1998). High temperature and the period to which the potato plants were exposed to it, did not positively influence their development. Contrary to these Kartha and Gamborg (1975), observed increasing in growth of cassava plant cultivated *in vitro*, when submitted to 35°C.

Chemotherapy

To investigate the effect of antiviral chemicals, ribavirin was used and the presence of virus was detected with DAS-ELISA test. The 20 mg L⁻¹ ribavirin was moderately effective in elimination of PVY from all replicates. As shown in (Table 3, 4), about of 30.0 and 42.9%, were PVY-free, respectively. These percentages were decreased to 6.7 and 22.2% when ribavirin was absent. Results showed by Simpkins *et al.* (1981), Dodds *et al.* (1989) and Griffiths *et al.* (1990) and indicated that addition of ribavirin (RBV) to the growth medium allowed satisfactory PVY elimination. The efficiency of RBV in the elimination of plant viruses is already well documented in the literature (Simpkins *et al.*, 1981; Chen and Sherwood, 1991; Lizarraga *et al.*, 1991; Fletcher *et al.*, 1998) and depends on the utilized concentration, host plant and type of infected tissue. This substance has a broad spectrum of action against DNA or RNA viruses infecting man, animals (Sidwell *et al.*, 1972) and plants (De Fazio *et al.*, 1980; Dawson and Lozoya-Saldana, 1984; Dawson, 1984; Hansen, 1984). *In vitro* culture and application of antiviral agents such as ribavirin (RBV); 5-azacitidine (5-AZA) and 3-deazauridine (3-DZD), have been successfully utilized in experiments involving potato cultivars toward the elimination of PVX, PVY, PLRV, Potato Virus S (PVS) and Potato Virus M (PVM) (Brown *et al.*, 1988; Kleinhempel *et al.*, 1990). This study indicated that PVY was not completely eliminated by the treatment of ribavirin alone and showed severe growth abnormalities. So that, the present study suggested that the electrotherapy or combination treatment with other means might be necessary.

Electrotherapy

When stem segments were connected directly to electrodes, most replicates exposed to 5/5, 5/10 and 10/5 mA min⁻¹ were confirmed as PVY-free (46.7, 57.1 and 64.7%, respectively). However,

at 10/10, 15/5 and 15/10 mA min⁻¹, these percent were increased to 73.3, 78.9 and 83.3%, respectively (Table 3). On the other hand, when the explants were fixed in the electrotherapy chamber, the percentages of virus-free plantlets was increased until to 83.3, 100 and 100% after treated with 10/10, 15/5 and 15/10 mA min⁻¹, respectively (Table 4). Electrical properties of plant tissue are considered in a wide range of physiological studies (Nelson, 1973). In this study, most replicates exposed directly to 15 mA were confirmed as PVY-free, which result 24.0% regeneration rate. The ratio of virus free reached to 83.3% when 15 mA/10 min was used (Table 3). This result was agree with Startseva *et al.* (1975). So that, result of this study clearly indicated that, directly electricity treatment seemed to affect the elimination of virus effectively. On the other hand, the regeneration rate was severely retarded when explants were fixed in electrotherapy chamber (3.2%) (Table 4). It was also noticed that, the temperature of potato stems treated with electric current was raised. The temperature may be suppressed viral activity but not inhibit the plant metabolism. Also, they suggested that, stem sap pH may be changed after electric shock treatment, which is related to inhibition of virus replication (Goldsworthy, 1987; Retivin and Opritov, 1992; Helliot *et al.*, 2007).

Table 3: Effect of directly electric current and ribavirin treatments on potato shoot-tip culture regeneration and PVY elimination

Elimination		Regeneration		Virus-free plantlets		
Treatments						
	Electric current					Therapy
Ribavirin	(mA min ⁻¹)	A	%	B	%	efficiency (%)*
With ribavirin	0/0	10/30	33.3	3/10	30.0	10.0
	5/5	12/35	34.3	8/12	66.7	22.9
	5/10	12/25	33.3	7/10	70.0	23.3
	10/5	10/32	31.3	8/10	80.0	25.0
	10/10	8/29	27.6	7/8	87.5	24.2
	15/5	7/28	25.0	7/7	100.0	25.0
	15/10	6/25	24.0	6/6	100.0	24.0
Without ribavirin	0/0	15/33	45.5	18/15	6.7	30.0
	5/5	15/31	48.4	7/15	46.7	22.6
	5/10	14/28	50.0	8/14	57.1	28.6
	10/5	17/30	56.7	11/17	64.7	36.7
	10/10	15/27	55.6	11/15	73.3	40.8
	15/5	19/30	63.3	13/19	68.9	43.6
	15/10	8/25	24.0	5/6	83.3	20.0

A: Plantlets regenerated/number of treated buds cultured. B: PVY-free plantlets/plantlets regenerated. * Therapy efficiency (TE) = A (%) × B (%) according to Lozoya-Saldana *et al.* (1996).

Table 4: Effect of indirectly electric current and ribavirin treatments on potato shoot-tip culture regeneration and PVY elimination

Elimination		Regeneration		Virus-free plantlets		Therapy efficiency (%)
Treatments						
Ribavirin	Electric current (mA min ⁻¹)	A	%	B	%	
With ribavirin	0/0	7/27	26.0	3/7	42.9	11.2
	5/5	9/33	27.0	6/9	66.7	18.7
	5/10	8/30	27.0	6/8	75.0	20.3
	10/5	7/29	24.1	6/7	85.7	20.7
	10/10	5/27	18.5	5/5	100.0	18.5
	15/5	4/30	13.3	4/4	100.0	13.3
Without ribavirin	15/10	0/31	0.0	0/0	0.0	00.0
	0/0	9/30	30.0	2/9	22.2	60.7
	5/5	9/29	31.1	5/9	55.6	17.2
	5/10	10/32	31.3	6/10	60.0	18.8
	10/5	8/29	27.6	5/6	75.0	17.2
	10/10	6/29	20.7	5/6	83.3	17.2
	15/5	5/28	17.9	5/5	100.0	17.9
	15/10	1/31	3.2	1/1	100.0	30.2

A: Plantlets regenerated/number of treated buds cultured. B: PVY-free plantlets/plantlets regenerated. * Therapy efficiency (TE) = A (%) × B (%) according to Lozoya-Saldana *et al.* (1996)

Combination of Chemotherapy and Electrotherapy

It was assumed that combination treatment with electricity as well as ribavirin might be effective in the virus elimination. To eliminate PVY from the potato variety used in this study, some axillary buds from the segment directly treated by electricity were excised and cultured in the medium supplemented with ribavirin (20 mg L⁻¹). At the end of culture, it was confirmed that PVY was completely eliminated in all replicates treated by electric currents 15/5 and 15/10 mA min⁻¹ then cultured in the medium with 20 mg L⁻¹ ribavirin (Table 3). On the other hand, the better result was obtained when explants were fixed in electrotherapy chamber and then cultured on the same medium, whereas PVY was completely eliminated at low electric currents (10 mA/10 min) (Table 4). This study confirmed that, PVY was eliminated in all replicates treated by electric current 15 mA for 5 min and cultured in the medium with 20 mg L⁻¹ ribavirin. Virus elimination indices further support the use of electrotherapy, together with the addition of ribavirin to the growth medium, as the best treatment for virus elimination in potato (Yi *et al.*, 2003). Other researchers such as Griffiths *et al.* (1990), observed similar results, virus concentration was reduced when the ribavirin was added to the medium and plants were submitted to thermotherapy. In present study, the plantlets freeing from virus were further propagated via., nodal cutting in the medium without ribavirin because plants were propagated better without ribavirin. The technique of *in vitro* cultivation of single nodal cuttings aimed to propagation virus-free plantlets, since it allows the production of plants in approximately 6 weeks, as compared to the 6 months required by meristem culture (Griffiths *et al.*, 1990).

Therapy Efficiency (TE)

The greatest TE, 43.6%, was obtained in ribavirin-free medium, following exposure to 15 mA for 5 min. The resultant rate is 63.3% regenerated plantlets of which, 68.9% were virus-free. On the other hand, after ribavirin addition, the highest TE value was 25%, whereas regenerated plantlets were decreased to 31.3%, of which 80.0% were virus-free (Table 3). The TE values were decreased when explants were treated by fixation in the electrotherapy chamber. The maximum value reached to 20.7% after exposure to 10 mA for 5 min, then decreased to 3.2% following exposure to 15 mA for 10 min and cultured in medium supplemented with ribavirin, which decreased the regenerated plantlets to zero (Table 4). The TE was affected strongly when thermal treatment was used; it was 25 and 12.3% after exposure to the first and second cycles, respectively (Fig. 1). Therapy efficiency TE was calculated by multiplying the percentage of regenerated plantlets X percentage of virus-free survivors. The data resulted from:

- High regeneration but few virus-free plantlets (Table 3, 45.5 and 6.7%, respectively)
- Moderate behavior in both observations (Table 3, 56.7 and 64.7%, respectively)
- Low percentage of regeneration, although all resulting individual plantlets were virus-free (Table 4, 3.2 and 100%, respectively)

Plant Regeneration

To investigate the effect of thermal, chemical and electrical shock treatments on the generation rate of plantlets during the tissue culture, the regeneration and growing pattern of each regenerated plantlets were observed. As indicated in Fig. 2, the rate of regeneration was retarded by thermal treatment. From 14 infected plants, which submitted to the first thermotherapy cycle, only 6 plants were survived. From 224 single nodal cuttings obtained, 166 plantlets were produced, corresponding to 74.1% regeneration. The rate of regeneration and growth of shoots were not greatly retarded by treatment of ribavirin (20 mg L⁻¹) supplemented in the culture media. The regeneration rates were decreased after using ribavirin from 45.5 to 33.3% and from 30.0 to 26.0% (Tables 3, 4), respectively. While electrical shock treatment slightly enhancement the regeneration rate until using

Table 5: Evaluation of plants resulted from virus free minitubers compared with virus infected plants

Potato seed sources	Plant height (cm)	Number of tubers/plant (g)	Average of weight tuber	Total yield/plant (g)
Virus-free minitubers	41.66a	10.67a	161.6a	1616.7a
Tubers infected with PVY	38.33b	8.67b	128.3b	1216.6b
Tubers infected with mix of PVY, PVX and PLRV	37.30c	7.30c	110.0c	866.7c
LSD _{0.05}	0.75	1.18	17.1	173.1

10 mA/10 min especially after directly treatment, then decreased after treatment with 15/5 and 15/10 mA/min. On the other hand, electrical shocked plant materials and then cultured on ribavirin supplemented media, resulted in a serious reduction in the regeneration rate, especially when 15 mA current was used (Table 3, 4). According to Wambugu *et al.* (1985), ribavirin treatment reduced growth rate in culture treated with 20 mg L⁻¹, which showed severe growth abnormalities such as chlorosis, stunting, root inhibition and cause deformation. The effect of the electric shock treatment on the regeneration rate was differed when treated explants were cultured on medium supplemented with ribavirin. This result not agreement with some earlier reports by Goldsworthy (1987). They reported that exposures to mild electric current, 5 to 10 mA for 5 min prior to *in vitro* culture, may improve regeneration of plant tissue. No morphological differences were observed between plants exposed to the different current treatments and controls.

Acclimatization and Minituber Production

After 3 weeks from beginning of acclimation, survivor plantlets were indexed serologically and then used as a mother plants, whereas 10 plantlets per pot were used. During 70-80 days growing period, each pot can produce about 45 minitubers and a weight range of 3-7 g per tuber.

Virus-Free Minitubers Evaluation

The results in Table 5 presented that the virus-free minitubers was the best than other infected seed tubers concerning for plant height, number of tubers/plant, average weight of tuber and also, total yield/plant characters. Potential losses are even greater in plants grown from PVY-infected tubers when compared with those grown from virus-free minitubere seeds. The virus-free minitubers gave the highest value than other infected tubers. The infected plant with PVY was milder symptoms than the infected plant with mixed viruses. The obtained results agreement with many researchers such as Spaar and Kleinhempel (1986), De Bokx and Huttinga (1981), Sangar *et al.* (1988), Hane and Hamm (1999) and Whitworth *et al.* (2006).

According to these results, efficiency of electrotherapy combined with ribavirin for eradication of potato viruses was higher. This technique must be combined with strong certification, which result plant materials for using to boost production of virus-free seed tubers. Also, using single node cuttings method especially in presence of ribavirin treatment was found to be technically easier and simpler than meristem culture. This study also demonstrated the usefulness of biologically test together with ELISA for direct detection of PVY from all samples. Growers in Egypt, used cutting seed tuber to pieces in-season of cultivation for potato production. At present, a tendency towards of a whole-tuber planting by using small size of seed tubers was achieved. This procedure can be prevented some pathogen (such as ring rot, bacterial wilt, PVX, potato spindle tuber viroid) transmitted by tuber-cutting. Also, due to planting whole small-size tuber for production, the labor for tuber-cutting could be saved. So that, development of seed potato production system characteristic of minituber multiplication must be expansion for virus-free seed potato.

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