STOCK INDEXING AND *POTATO VIRUS Y* ELIMINATION FROM POTATO PLANTS CULTIVATED *IN VITRO*

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ABSTRACT: Potato cultivars (*Solanum tuberosum* L.) have shown degeneration or run out caused by viruses after several cycles of propagation using seed tubers from commercial fields. This work reports the occurrence of single and mixed infections of four potato viruses in Paraíba–Brazil and presents a method for *Potato virus Y* (PVY) elimination, by using thermo-and chemotherapies. Plants of potato cv. Baraka were tested by direct antigen coating ELISA. Antisera against PVY, *Potato virus X* (PVX), *Potato virus S* (PVS), and *Potato leafroll virus* (PLRV) were used. Materials with positive reaction to PVY were treated for virus elimination. Single node cuttings (1.0 cm length) were excised and inoculated in Murashige & Skoog (MS) medium, supplemented with 1.0 mg L^{-1} of kinetin, 0.001 mg L^{-1} of naphthalene acetic acid (NAA) and 0.1 mg L^{-1} of gibberellic acid (GA_3). The thermotherapy at approximately 37ºC, during 30 and 40 days, resulted in 20.0 and 37.5% PVY elimination, respectively. Chemotherapy was undertaken with Ribavirin (RBV), 5-Azacytidine (AZA), and 3-Deazauridine (DZD). The RBV showed the highest rate of virus eradication, with 55.5% virus-free plants. Simultaneous thermo and chemotherapy had higher efficiency for the elimination of PVY, reaching rates of healthy plants of 83.3% with RBV, 70.0% with AZA, and 50.0% with DZD.
Key words: *Solanum tuberosum*, virus-free clones, thermotherapy, chemotherapy

INDEXAÇÃO DE MATRIZES E ELIMINAÇÃO DO *POTATO VIRUS Y* EM PLANTAS DE BATATA CULTIVADAS *IN VITRO*

RESUMO: Cultivares de batata (*Solanum tuberosum* L.) têm mostrado degenerescência causada por vírus após ciclos sucessivos do uso de tubérculos de campos comerciais como material propagativo. Este trabalho verifica a ocorrência de infecção simples e mista de quatro vírus da batata na Paraíba e apresenta adequação da técnica de cultivo *in vitro* para obtenção de material livre de *Potato virus Y* (PVY), incluindo uso de microestacas, termo e quimioterapia. Plantas de batata do cv. Baraka foram submetidas à indexação sorológica pelo teste "direct antigen coating" ELISA. Utilizaram-se antissoros contra o PVY, *Potato virus X* (PVX), *Potato virus S* (PVS) e *Potato leafroll virus* (PLRV). Materiais com reação positiva para PVY foram submetidos a tratamentos visando à eliminação viral. Microestacas (1,0 cm de comprimento) com uma gema foram excisadas e inoculadas em meio nutritivo de Murashige & Skoog (MS), suplementado com 1,0 mg L^{-1} de cinectina, 0,00l mg L^{-1} de ácido naftaleno acético (ANA) e 0,1 mg L^{-1} de ácido giberélico (GA_3). Termoterapia a 37ºC, durante 30 e 40 dias, promoveu a eliminação do PVY em 20,0 e 37,5% no material testado, respectivamente. A quimioterapia foi realizada com Ribavirin (RBV), 5-Azacitidina (AZA) e 3-Deazauridine (DZD). O RBV apresentou os melhores índices de erradicação de vírus com a obtenção de 55,5% de plantas sadias. Tratamentos simultâneos de termo e quimioterapia mostraram maior eficiência na eliminação viral, atingindo um percentual de plantas sadias da ordem de 83,3 com RBV, 70,0 com AZA e 50,0 com DZD.
Palavras-chave: *Solanum tuberosum*, limpeza clonal, termoterapia, quimioterapia

INTRODUCTION

Seed tuber quality is an extremely important factor for potato yield. Since it is a vegetatively-propagated plant, fungal, bacterial and, particularly viral disease, agents are easily transmitted through the tubers (Truta, 1997). Viral diseases are, for the most part, responsible for degeneration, characterized by a decrease in vigor, productivity, and resistance to diseases of potato cultivars after successive cultivation from the same lot of tubers (Silberschmidt, 1937; Sangar et al., 1988).

In Northeastern Brazil, the occurrence of potato virus, including *Potato virus Y* (PVY), was observed in commercial fields in the states of Pernambuco (Pio-Ribeiro et al., 1994; Marques et al., 1996; Assis Filho et al., 1997) and Paraíba (Pio-Ribeiro et al., 1997), developing as single and mixed infections. PVY is the type species of the genus *Potyvirus*, in the family *Potyviridae*

(Kitajima et al., 1997). It occurs as long, flexuous particles measuring 730 x 11 nm, containing single-stranded, positive polarity RNA (De Bokx & Huttinga, 1981). 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 (Silberschmidt & Kramer, 1942; Souza Dias & Iamauti, 1997).

One of the most viable methods for obtaining virus-free stocks from propagative material that comes from infected plants is viral eradication by using tissue culture techniques, aided or not by thermo-and/or chemotherapies (Mellor & Stace-Smith, 1970). These methodologies allow quick propagation of plant material, producing healthy plants from a single individual in a short period of time, regardless of location or season of the year.

Chemotherapy directed to plant viral diseases has evolved significantly. Not only substances that inhibit viral replication, but also induce resistance, have been discovered. The most frequently studied compounds are antimetabolites, substances capable of blocking the virus nucleic acid synthesis (Vicente & De Fazio, 1987). These compounds, both natural and synthetic, express an antiviral effect, but none of them present a satisfactory selective action that would enable them to be used in specific prophylaxis and in large-scale therapy of plant viral diseases (Hansen & Lane, 1985).

Antivirals have been utilized for *in vitro* cultivation of plants, as a therapeutical supplement in integrated control programs targeted at plant viral diseases. Compounds such as Ribavirin (RBV) (1-β-D-ribofurasonyl-1,2,4 triazone-3-carboxamide) (Hansen & Lane, 1985; Griffiths et al., 1990; Chen & Sherwood, 1991; Toussaint et al., 1993; Prasada Rao et al., 1995), 5-Azacytidine (AZA) (4-amino-1-β-D-ribofuranosyl-5-triazin-2[1H]-one) and 3-Deazauridine (DZD) (4-hydroxi-1-β-D-ribofuranosyl-2 [1H] pyridone) (Dunbar et al., 1993) have been successfully utilized for virus elimination in economically important crops, such as potato, peanut (*Arachis hypogaea* L.), apple (*Pyrus malus* L.) and *Prunus* spp.

The present work was developed to perform indexing of a potato stock with regard to viruses and to adjust *in vitro* cultivation techniques for PVY elimination, with the use of single node cuttings, and joint utilization of thermo-and/or chemotherapies.

MATERIAL AND METHODS

Serologic indexing of potato material

A preliminary test was carried out with foliar samples of 143 plants of cv. Baraka that exhibited viral infection symptoms, in a commercial field in Paraíba, Brazil (7º3'16"S and 36º3'10"W), 40 days after planting, by using an antisera mixture against PVY, *Potato virus X* (PVX), *Potato virus S* (PVS) and *Potato leafroll virus* (PLRV), each at the concentration of 1:1000 (v/v). Samples showing positive reaction were submitted to another test with the distinct antisera to determine viral identity and to identify and mark in the field plants having different types of infection.

The presence or absence of viruses in the studied material at different stages of the work was determined by direct antigen coating ELISA (DAC-ELISA), according to the basic methodology presented by Gugerli & Gehriger (1980) and Hobbs et al. (1987), with modifications in the preliminary test, relative to the use of an antisera mixture. After adding the substrate, the plates were maintained at room temperature time enough to allow visual observation of the colorimetric reaction. The reaction was halted by adding 40 μL of sodium hydroxide 3 mol L^{-1} per well, followed by reading at the 405 nm wavelength.

Obtaining PVY-infected potato stock

At harvest, tubers from 30 plants that reacted positively to PVY were collected. The yield from each plant was individually stored in cold chamber during eight months and then planted in a greenhouse, to obtain material for the *in vitro* isolation. After sprouting, plantlets were indexed by using the distinct antisera to certify the occurrence of single infection by PVY in the selected material. Since plenty of infected leaves were available, dissected tissue was harvested to be used as positive control in subsequent serological tests.

In vitro cultivation of potato from single node cuttings

The culture medium for growing potato bearing viral infection was prepared with macro and micronutrient salts and vitamins of the MS medium (Murashige & Skoog, 1962), supplemented with 1 mg L^{-1} thiamine, 100 mg L^{-1} inositol, 2 mg L^{-1} glycine, 30 g sucrose; 8.0 g agar; 0.001 mg L^{-1} auxin (naphthalene acetic acid - NAA); 1.0 mg L^{-1} cytokinin (6-furfurylaminopurine - Kinetin), and 0.1 mg L^{-1} gibberellin (gibberellic acid - GA_3). The pH of the medium was adjusted to 5.7 and the agar was melted at mild heating, and immediately distributed into test tubes (20 x 150 mm Pyrex), at the rate of 10 mL per tube, which were then sealed with aluminum foil and autoclaved.

Single node cuttings around 1.0 cm in length containing one bud were taken from the PVY-infected stock plants grown in a greenhouse. The single node cuttings were immersed in ethyl alcohol 70% for 30 seconds, then transferred to a 2% hypochlorite solution for 10 minutes and washed three times, sequentially in sterilized distilled water (ADE). Explants were individually cultivated and maintained in a growth room, under a temperature regime of 25 ± 2ºC and a 16-hour photoperiod provided by fluorescent bulbs with light intensity of 110 μmols m^{-2} s^{-1}.

Thermotherapy

Thermotherapy was carried out in two cycles. Firstly, 13 infected plants, recently established *in vitro*,

from stock plants, showing root growth and emission of leaves, were submitted for 40 days to a continuous light regime and temperature of 37 ± 2ºC and then indexed. Several transplantings were performed from plants that remained infected, by using single node cuttings grown in culture medium and maintained at 25 ± 2ºC, until plants reached between 8 and 10 cm, originating a great number of infected plants, according to performed indexings, which were utilized in subsequent stages of the work. During the second cycle, 30 plants recently established *in vitro* were transferred to an incubator at 37 ± 2ºC, 16-hour photoperiod, and light intensity of 110 μmols m^{-2} s^{-1}, where they remained for 30 days.

Chemotherapy

Single node cuttings from *in vitro* transplants, infected by PVY, were grown in culture medium set up as a control and in a medium supplemented individually with antivirals RBV, AZA and DZD, at concentrations of 20 mg L^{-1}, 20 mg L^{-1} and 30 mg L^{-1}, respectively. Chemotherapy agents were autoclaved together with the culture medium at 120ºC, 1atm, for 15 minutes, or sterilized with millipore filters (0.22 μm), and added to the medium before solidification. Twenty-one single node cuttings were utilized per treatment and incubated for 60 days.

Chemo- and thermotherapies

Single node cuttings from PVY-infected plants, transplanted *in vitro*, were grown in control medium and medium + antiviral (RBV, AZA or DZD) sterilized by ultrafiltration, and incubated at 37 ± 2ºC, with a 16-hour photoperiod, and light intensity of 110 μmols m^{-2} s^{-1}, where they remained for 30 days. Fifteen single node cuttings were utilized per treatment.

Acclimation

Plants submitted to chemotherapy, regenerated with roots and a well developed aerial part, were removed from the culture medium and their roots were washed in running water. These plants were acclimated in pots containing a mixture of soil, vermiculite and organic matter, in the 2:2:1 proportion, and kept under a transparent polystyrene cover, for two weeks. The plants remained in an environment without incidence of direct sunlight during this period. In the first weeks of acclimation, plants were irrigated daily with water and Hoagland nutrient solution. Survivor plants were indexed serologically.

RESULTS AND DISCUSSION

Stock indexing and *in vitro* cultivation of PVY-infected plants

When the mixture of four antisera was utilized in the DAC-ELISA test, 90 and 53 plants, presented positive and negative reaction, respectively. The utilization of specific antisera allowed identification of individual and simultaneous presence, in several combinations, of the PVY, PVX, PVS and PLRV viruses in plants showing positive reaction (Table 1). This result supports the information on the legitimate use of polyvalent antisera, formed by the mixture of specific antisera, to obtain healthy material, just by verifying the presence or absence of viruses, without determination of the viral identity. Using the double antibody sandwich ELISA test for viral detection in potato, Truta (1997), observed the same sensibility when using individual or mixed antisera; therefore, any of the forms can be adopted satisfactorily for indexing.

PVY was detected in 60 out of 143 indexed potato plants; of which 34 showed single infection and 26 mixed infection. From the material bearing single infection, 13 infected plants were obtained *in vitro*.

The temperature and photoperiod utilized during the incubation were favorable to the development of the potato plants. Conditions were similar to those utilized by Alconero et al. (1975) for sweet potato (*Ipomoea batatas* (L.) Lam.); Dunbar et al. (1993) for *Arachis* hybrids; and Sarkar & Naik (1998) and Jayasree et al. (2001) for potato.

Thermotherapy of plants multiplied *in vitro* by single node cuttings

From the 13 infected plants established *in vitro* and submitted to the first thermotherapy cycle (40 days), eight survived and were propagated by single node cut-

Table 1 - Serologic analysis of potato plants, indexed through a mixture of antisera and specific antisera, by direct antigen coating ELISA.

Reaction to antisera mixture	Viruses[1] detected by specific antisera	Number of plants per reaction to specific antisera
+	PVY	34
+	PVY, PVX	3
+	PVY, PVS	5
+	PVY, PLRV	2
+	PVX	2
+	PVX, PVS	8
+	PVX, PLRV	1
+	PVS	6
+	PVS, PLRV	6
+	PLRV	3
+	PVY, PVX, PVS	1
+	PVY, PVS, PLRV	6
+	PVX, PVS, PLRV	3
+	PVY, PVX, PVS, PLRV	10
-	---	53

[1]PVY = *Potato virus Y*; PVX = *Potato virus X*; PVS = *Potato virus S* and PLRV = *Potato leafroll virus*.

tings. The indexing of somaclones showed that three survivors were virus-free, indicating that a 37.5% efficiency was achieved through this PVY elimination process. After *in vitro* transplantings from five infected plants, 300 single node cuttings were obtained and from these, 231 plants were produced, corresponding to a 77.0% regeneration percentage. Of the 30 plants that were submitted to the second thermotherapy cycle, (30 days), six produced PVY-free progenies, thus achieving a virus elimination index of 20.0%.

Using temperature of 37°C, for 30 or 40 days, can be considered adequate for virus elimination in potato plants cultivated *in vitro* from single node cuttings. Temperatures from 34 to 40°C for periods ranging from days to weeks are efficient for viral eradication (Betti, 1991). Thomson (1956) and Leonhardt et al. (1998) eliminated PVY from *in vitro* potato plants by using temperatures between 35 and 38°C. The percentage of virus elimination with thermotherapy can be increased by the simultaneous adoption of other techniques (Hollings, 1965; Stace-Smith & Mellor, 1968; Slack, 1980).

High temperature and the period to which the potato plants were exposed to it did not positively influence their development, contrary to results obtained by Kartha & Gamborg (1975), who observed plant growth increase in cassava cultivated *in vitro*, when submitted to 35°C. The interaction between environmental factors during the growth and conservation of *in vitro* potato plants can be decisive, since it is a photosensitive crop. The 16-hour photoperiod has been utilized in potato (Russo & Slack, 1998; Jayasree et al., 2001) and grape (*Vitis* spp.) experiments (Leonhardt et al., 1998).

The regeneration index of potato plants observed in this work, by utilizing single node cuttings, can be considered high as compared to those obtained by Kassanis & Varma (1967), 10.5%; Stace-Smith & Mellor (1968), 37.0%; and Sip (1972), 42.0%. At 30 days from the beginning of cultivation the material reached a stage where plants had a well-developed aerial part and good root system. The technique of *in vitro* cultivation of single node cuttings in projects aimed at virus elimination, is extremely attractive from a practical standpoint, since it allows the production of plants in approximately six weeks, as compared to the six months required by meristem culture (Griffiths et al., 1990).

The *in vitro* cultivation of infected material showed that it is viable to maintain the viral isolates studied under these conditions. This method allows economy of time and space, overcomes problems of contamination by other viruses and prevents exotic viruses from escaping (Pio-Ribeiro et al., 1993).

Potato plant chemotherapy

Control plants grew better and differed ($P < 0.01$) from all other treatments (Table 2). Smallest heights were observed in treatments containing autoclaved and filtered RBV. The use of antivirals influenced plant survival, as it provided higher values than the control, but there were no differences among the tested antivirals or between forms of sterilization. Toxicity to plants and antiviral activity were more intense when filtered agents were used, relatively to those autoclaved together with the culture medium, thus determining growth or causing plant death, as verified by Griffths et al. (1990).

The utilized antiviral concentrations and their form of application to the growth medium allowed satisfactory PVY elimination indices, similarly to what was observed by Dodds et al. (1989); Griffths et al. (1990); Simpkins et al. (1981) and Betti (1991). The greatest proportion of healthy, single node cuttings was obtained with RBV, both filtered and autoclaved, resulting in PVY eradication in the order of 55.5 and 41.7%, respectively. A high percentage of elimination was also verified for the filtered and autoclaved AZA antiviral, at 37.5% and

Table 2 - Effect of antiviral agents on the development of *in vitro* potato plants and virus elimination, after 60 days of cultivation[1].

Treatment[2]		Survival	Virus elimination	Mean plant height[4]
Antiviral[3]	Type of sterilization			
		%		cm
Control	No antiviral	0.0	76.1	10.0 a
Ribavirin	Filtered	55.5	42.8	3.0 e
Ribavirin	Autoclaved	41.7	57.1	4.0 d
5-Azacytidine	Filtered	37.5	33.3	6.0 c
5-Azacytidine	Autoclaved	33.3	42.8	9.0 b
3-Deazauridine	Filtered	28.6	28.5	5.0 cd
3-Deazauridine	Autoclaved	28.6	28.5	8.0 b

[1]Murashige and Skoog (1962) nutritive medium, modified.
[2]Tewnty one single node cuttings of potato were grown, having 1.0cm in length and one bud, per treatment.
[3]Agents supplemented to the medium, at concentrations of 20 mg L^{-1} (Ribavirin and 5-Azacytidine) and 30 mg L^{-1} (3-Deazauridine).
[4]Means followed by a common letter do not differ by Tukey test at 1%

33.3%, respectively. Treatments containing DZD showed 28.6% elimination, whether filtered or autoclaved. There were differences between results obtained in the controls and in treatments containing antiviral compounds ($P < 0.01$). The number of infected single node cuttings did not differ among the three antiviral agents tested and between the two forms of sterilization.

The efficiency of RBV in the elimination of plant viruses is already well documented in the literature (Simpkins et al., 1981; Vicente & De Fazio, 1987; Chen & 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 (Dawson, 1984). *In vitro* culture and application of antiviral agents such as RBV, 5-AZA and 3-DZD have been successfully utilized in experiments involving potato cultivars toward the elimination of PVX, PVY, PLRV and *Potato virus M* (PVM) (Brown et al., 1988; Kleinhempel et al., 1990), and PVS (Kleinhempel et al., 1990).

Simultaneous application of thermo and chemotherapy in potato plants

Of the 15 single node cuttings utilized in each treatment, 12 plants submitted to RBV and thermotherapy, and ten plants in each one of the other treatments were regenerated, and no difference was obtained ($P > 0.05$). Overall, plants treated with RBV showed less vigor than those submitted to the action of AZA and DZD. The development of root and shoot was sluggish, probably because of the toxic effect to plants caused by the chemotherapy agents, as observed by Bittner et al. (1987) for potato; Deogratias et al. (1989) for cherry (*Prunus* spp.), and Hansen & Lane (1985) for apple.

Joint effects of thermo and chemotherapies, applied to *in vitro* potato plants, were highly efficient in eliminating PVY. Treatments that included RBV, AZA and DZD resulted in PVY elimination from 83.3; 70.0 and 50.0% of the plants, respectively. Among plants submitted to thermotherapy without antiviral treatment, 30.0% were PVY-free, differing from RBV ($P < 0.05$). These percentages are higher than those obtained by Dunbar et al. (1993), who eliminated *Peanut mottle virus* (PeMoV), of the genus *Potyvirus*, family *Potyviridae*, from 36.0; 50.0; and 24.0% of peanut plants, respectively, with the three tested antiviral agents, at similar concentrations.

Virus elimination indices further support the use of thermotherapy, together with the addition of antiviral agents to the growth medium, as the best treatment for virus elimination in potato (Fletcher et al., 1998; Dodds et al., 1989). Other authors such as Griffiths et al. (1990), observed similar results, reducing the concentration of virus when the antiviral RBV was added to the medium and plants were submitted to thermotherapy.

Acclimation

Among plants submitted to chemotherapy and transferred to a substrate containing soil, vermiculite and organic matter, two out of five were successfully acclimated. Indexing of the acclimated plants showed that both were virus-free, confirming results obtained in tests conducted previously with leaf samples harvested from the same plants cultivated *in vitro*.

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