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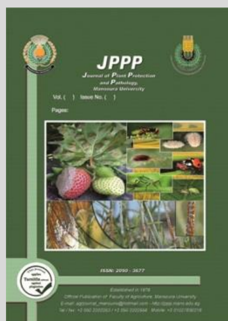
Impact of Chemotherapy and Thermotherapy Treatments on The Presence of Potato Viruses Pvy, Pvx and Plrv in Tissue-Cultured Shoot Tip Meristem

Shoala, T.[1*]; Kh. E. Eid[2] and I. A. I. EL-Fiki[2]

[1]Misr University for Science and Technology, College of Biotechnology, Environmental Biotechnology Department, Al-Motamayez District - 6th of October, Egypt.
[2]Plant Pathol., Dept., Fac. .of Agric., Moshtohor, Benha Univ



ABSTRACT

Potato tubers (*Solanum tuberosum* L.) is considered as one of the most essential vegetable crops worldwide, The most communal viruses attacking potato all over the world are potato leaf roll virus (PLRV), potato virus Y (PVY) and potato virus X (PVX). Our current study aimed to eradicate potato viruses PVY, PVX and PLRV through shoot tip meristem via tissue culture combined with thermotherapy and chemotherapy. Sprouts of potato cultivars *ex.* Spunta, Selana, Diamond and Cara were used as biological material for investigating their performance using shoot tip meristem. During multiplication stage, the resulted plantlets were subjected to ribavirin (50 mg/L), while the untreated plantlets served as control. In general, the control treatment (0.0 mg/L) provided the best results, while ribavirin (50 mg/L) gave reverse effect on the estimated vegetative parameters (shoot length (cm), number of leaves and number of shoots). Detection of the presence and absence of PVY, PVX and PLRV using DAS-ELISA technique indicated that, thermotherapy and chemotherapy treatments for shoot tip meristem culturing minimized the occurrence percentage of PVX, PVY and PLRV in all tested potato cultivars compared to positive control. The results of RT-PCR revealed that, the amplicons of amplified fragments and primed by PVX-, PVY- and PLRV-specific sense and antisense primers were appeared only in the positive control (lane 1 at ~750, 801 and 548 bp), respectively, while, no amplicons appeared in the rested treatments. In conclusion, shoot tip meristem followed by thermotherapy and chemotherapy treatments enhanced the growth of potato plantlets and suppressed potato viruses.

Keywords: Potato (*Solanum tuberosum* L.), Shoot tip meristem culture, Thermotherapy, Chemotherapy, ELISA test, RT-PCR test.

INTRODUCTION

Potato tubers (*Solanum tuberosum* L.) have been eaten since time immemorial. The current cultured potatoes have been spread 160 countries worldwide from their origin the Andes of South America. Potato production of Egypt exceeds 4.3 million Mega grams from 163,939 hectare in 2017 (FAOSTAT, 2017). So, it is well known that potato is considered as one of the most valuable vegetable crops all over the world as it follows rice and wheat as a food for human consumption (Camire *et al.,* 2009). Potato is a high-yielding carbohydrate-rich crop; energy source with slight fat, its protein constituents is justly low but has an exceptional biological importance of 90–100, an excellent source of potassium, vitamins C and B6. Moreover, many composites in potatoes subsidize antioxidant activities like flavonoids, carotenoids and phenolic compounds.

Furthermore, potato is a cholesterol-free vegetable crop (Brown, 2005). Accordingly, it can be used as dietary supplements and as protective agents against common tumor types such as breast and prostate cancer.

Unfortunately, it has been reported that potato could be infect by many viral and viroidal diseases like potato leaf roll virus (PLRV), potato virus A (PVA), potato virus M (PVM), potato virus S (PVS), potato virus X (PVX), potato virus Y (PVY) and potato spindle tuber viroid (PSTVd). The most communal viruses infecting potato all over the world are PLRV, PVY and PVX (Abou-Jawdah *et al.,* 2001). It is well established that viral diseases cannot be controlled or managed using chemicals or even antibiotics. Using of virus-resistant potato cultivars or free potato tuber production is desired only for escaping losses of crop production. Currently, shoot tip meristem culture is the best methodology to produce plant free virus (Loebenstein *et al.,* 2001; Danci *et al.,* 2011). Since meristematic tip is the region of actively dividing cells, positioned at the apices of both shoots and roots, that remain in the dividing state during the vegetative stage, developing new tissues and organs (shoots, leaves, roots), therefore, meristematic tip have the ability of generating whole plants *In-Vivo* and *In-Vitro* (Danci, 2009).

Our current research aimed to throw a light on the possibility of producing free-virus potato plants using shoot tip meristem technique. Moreover, diagnosis of potato plant viruses PLRV, PVX and PVY in potato plantlets.

MATERIALS AND METHODS

Source of plant materials

Infected potato tuber cultivars *ex.* Spunta, Selana, Diamond and Cara with PVX, PVY and PLRV were kindly

[*] Corresponding author.
E-mail address: tahsen.shoala@must.edu.eg
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obtained from Virology Department, Plant pathology Institute, Agricultural Research Center, Giza, Egypt. All the following experiment steps were done under laboratory conditions.

Potato sprouting and starting stages for potato cultivars Spunta, Selana, Diamond and Cara were sterilized superficially for 2 minutes in 1.5% sodium hypochlorite, and then dipped 3 times in sterilized distilled water, then desiccated between two pasteurized filter papers. Conferring to the method defined by Espinoza et al. (1984) with some modifications, five tubers of each potato cv. were placed in plastic plates and then, incubated at 42^0C for one month for sprouting. The high temperature improved the production rate of virus-free materials.

Potato shoot tip meristem technique

For potato plantlets production, Murashige and Skoog's medium was prepared and used as meristem tip culture media (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose and 8 g/L agar and without any supplement of growth regulators. The pH was adjusted to 5.8 before sterilization at 1.5 Kg/cm^2 for 20 min.

Afterward, the 3-4 apical nodal shoot tips (explants) of each potato cv. were secluded and disinfected superficially with 70% (v/v) ethanol for 30 sec. followed by 1.5% (v/v) sodium hypochlorite solution for 1 min, removed, then dipped 3 times in sterilized distilled water and then, desiccated between two pasteurized filter papers. The ideal size of meristem is ranging from 0.1-0.2 mm (meristematic cap with few primordial leaves).

The apical buds were collected and sequestered from potato tuber buds then disinfected by dipping in 70% ethanol for several seconds, subsequently soaking in mercuric chloride 0.1% for 2 minutes and washed 3 times with sterile, distilled water. The tip and sub-tending leaf primordial detached and the meristems isolated under laminar airflow cabinet using dissecting microscope. Afterward, shoot tip meristem transferred into the prepared glass jars containing 33 mL of MS medium. The jars were incubated in a growth room at 20-21^0C and 70% humidity for four weeks under 16hrs photoperiod with 2000 lux intensity. The resulted explants of each tested potato cultivar gave raised from the aforementioned step were heat-treated according to the thermotherapy-based methods described by Panattoni et al. (2013) where, these explants were heat-treated with 42°C for 15 days and then incubated in growth cabinet (21-22°C).

Four weeks later, the developed plantlets were shifted into new jars containing 33 ml MS medium supplemented with adenine sulfate (10 mg/L), calcium pantothenate (5.0 mg/L), GA3 (1.0 mg/L), sucrose (30 g/L), agar (8.0 g/L). Then, pH was adjusted to 5.8 before autoclaving at 1.5 Kg/cm2 for 20 min. Ribavirin (50mg/l) was filter-sterilized using a Millipore filter (0.22 µm) and supplemented into the medium after autoclaving.

The cultured jars were then randomly placed in the growth chamber having temperatures and humidity as mentioned before. The procedure was sustained and repeated three times until complete plantlets were obtained in adequate numbers (40 jars represented 5 replicates for each cultivar). The initiated multiplied plantlets were maintained for 30 days for shoot proliferation. The fully developed plantlets were daily checked for any contamination. Six weeks later, shoot length (cm), number of leaves and number of shoots were recorded. The bulk of resulted plantlets treated with ribavirin (50 mg/L) (Panattoni *et al.*, 2013; Faccioli, 2001) and therapeutic treatment (42°C) for 15 days in addition to control treatment of each tested potato cultivar were gathered and used for preparation of plant extract acquired for the DAS-ELISA test.

Detection of PVX, PVY and PLRV occurrence using DAS-ELISA

Antiserum, antibody conjugated with alkaline phosphatase (IgG conjugate) and positive control for PVX, PVY and PLRV were purchased from LOEWE Co located in Mühlweg 2a, Sauerlach, Germany. DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay) procedure was performed according to the protocols described by Clark and Adams (1977). Microtiter plate wells (8 x12) flat-bottom wells of c. 400 µl/well) were coated with antiserum diluted in carbonate buffer (pH 9.6) according to the supplier's specifications. IgG used in the present experiment was diluted in the coating buffer (carbonate buffer) in the proportion of 1:200. Plates were incubated for 4 hrs at 37^0C and then, plantlet extracts of each cultivar were applied on these wells. The plant extracts were prepared with a hand-held grinder in a maceration buffer containing phosphate buffered saline (pH 7.4) with 0.5 ml/L Tween 20 and 2% polyvinyl pyrrolidone. After adding the crude plant extract, the virus was detected by the corresponding antibody conjugated with alkaline phosphate diluted in conjugate buffer (pH 7.4) according to the supplier's specifications. Plates were washed with phosphate buffered saline (pH 7.2-7.4) at each stage. Absorbance at 405 nm was measured using a ELISA Reader (Model: PR1100 reader) at 15 min., 30min. and 1 h post incubating the sample with the substrate p-nitrophenyl phosphate (1 mg/ml, pH 9.8). A sample was considered positive if the absorbance was at least three times greater than that of the healthy control plant (negative control).

Detecting of PVX, PVY and PLRV occurrences using RT-PCR:

RNA extraction

Total RNA has been extracted from infected tissue cultured potato plantlets using the EZ-10 Spin Column Total RNA Minipreps Super kits, then RNA purified according to the protocol of Thermo Scientific GeneJET Plant RNA Purification Mini Kit. Then, extracted RNA has been applied as a template for cDNA synthesis. A fragment of PLRV with expected size of (~548 bp) has been amplified (Shojaei, 2009). Fragments of PVX with expected size of (~700 bp) and PVY with expected size of (~801 bp) have been amplified (Shalaby *et al*., 2002).

RT-PCR Detection

Molecular diagnosis to detect the presence or absence of PLRV, PVY and PVX viruses, according to the kit used for the RT-PCR is: (VersoTM) one step RT PCR kit (Thermo scientific, cat. No. AB 1454/V6/1107). The extracted RNA was applied as the initial template for one tube RT-PCR amplification master mix using Verso TM one step RT- PCR kit utilizing specific primers for the PLRV, PVX and PVY.

PLRV F: 5'AATAG AA TTC TAAT GAG TAC GGT CGTGGTTARAGG3' and PLRV R: 5' AAA ACC ATGGCTATYTGGGGTTYTGCARAGCYAC3'.

PVX-F: 5' GAT AGA AT TCA GAT GAC TACA CCA GCCAACACC3' and PVX-R:5' TACG CGTCGGTT CCATGGACGTAGTTATGGTGG3'.

PVYF:5'TCAAGGATCCGCAAATGACACAAT TGAGCA GG3' and PVY-R: 5'AGAGAGAATT CATCACAT GTTCTTGACTCC 3'.

RT-PCR was achieved in 25 µl total volume containing 4.75 µl of nuclease - free water, 3ng/µl of total RNA, 12.5 µl of one step PCR master mix,3 µl of 10 µM of each primer,0.5 µl Verso enzyme mix and 1.25 µl RT-Enhancer. RT reaction initiated with incubation at 50 C for 15 min, followed by denaturation at 95C for 5min. The amplification reaction was performed through 35 cycles in Gradient thermal cycler (Biometra, Germany) starting with denaturation at 94°C for 30 sec, primer annealing at (50-55°C for 30 sec and extension at 72°C for 2 min. Final extension at the end of the 35th cycle was performed at 72°C for7 minutes.

Gel electrophoresis preparation :7 µl of RT-PCR product were examined on 1% agarose gel in TBE buffer (89 mM Tris-HCL, pH 8.5) at 120 volt.100 bp sharp DNA ladder marker (RBC) was loaded to define the size of RT-PCR products. Gels stained with ethidium bromide 10 µg/ ml and imagined using gel-documentation system (Bio-Rad, GelDoc XR) (Sambrook *et al*., 1989).

RESULTS AND DISCUSSION

Results

The main objective of this study was detecting the existence of PVX, PVY and PLRV in the resulted and multiplicated potato plantlets *in vitro* through reliable and sensitive protocols.

Effect of Ribavirin on potato plantlets

Potato tuber cultivars ex. Spunta, Selana, Diamond and Cara potato tubers were chosen and prepared for sprouting. The resulted sprouts for each potato cultivar were used and tissue-cultured into MS medium. Potato sprouts were exposed to heat treatment (42°C) to suppress the presence of potato viruses PLRV, PVY and PVX. ribavirin (50mg/L) was added to the MS medium during the multiplication stage. In general, data presented in figure (1) showed that MS-media amended with ribavirin (50mg/L) minimized the measured vegetative parameters, while the MS-media amended with ribavirin (0.0 mg/L) (control treatment) produced the highest values for shoot no., shoot length (cm) and leaves no. for each tested potato cultivar. In this respect, Selana cultivar exhibited the highest values of assessed average measurements, leaves number, shoot length and shoot numbers (14, 8.5 cm. and 5.5 shoots) respectively compared to (7, 7 cm and 4 shoots) in the treated MS medium with ribavirin (50 mg/L). On the other hand, Spunta cultivar recorded average measurements, leaves number, shoot length and shoot numbers (13, 7 cm. and 4 shoots) respectively compared to (7, 5 cm and 4.5 shoots) in the treated MS medium with ribavirin (50 mg/L).

While, Cara cultivar showed that average parameters of leaves number, shoot length and shoot numbers (10, 7.5 cm. and 5 shoots) respectively compared to (6, 6 cm and 4 shoots) in the treated MS medium with ribavirin (50 mg/L).

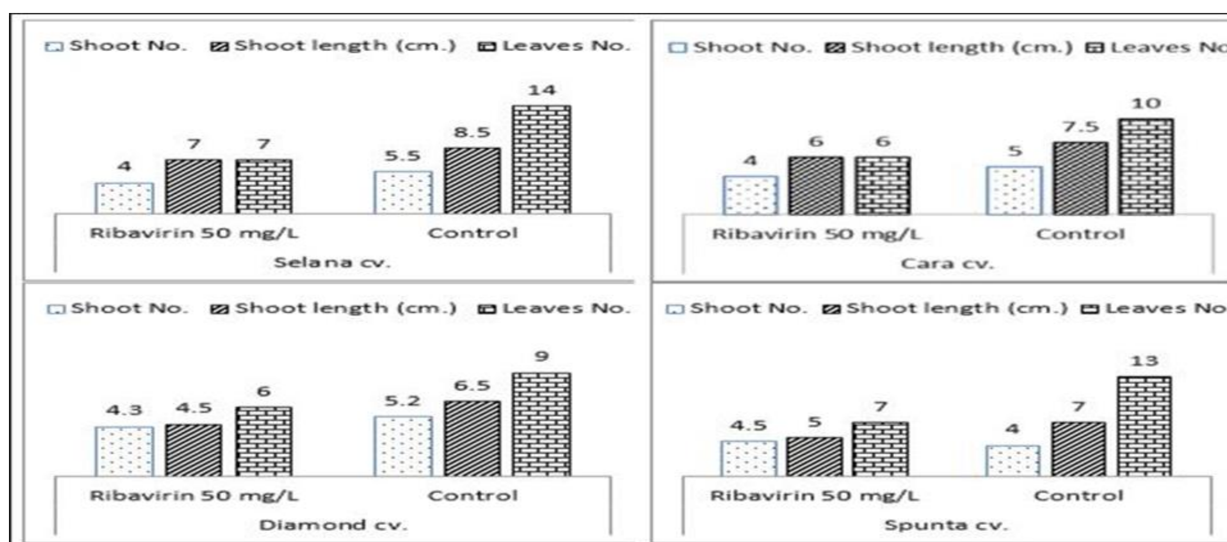

Fig .1. Effect of Ribavirin (50 mg/L) on some vegetative parameters of resulted tissue-cultured potato cultivars.

Controversy, Diamond cultivar recorded average parameters of leaves number, shoot length and shoot numbers (9, 6.5 cm. and 5.5 shoots) respectively compared to (6, 4.5 cm and 4.3 shoots) in the treated MS medium with ribavirin (50 mg/L).

Detection of PVX, PVY and PLRV using DAS-ELISA

The resulted plantlets of the four tested potato cultivars were inspected for the occurrence of PVX, PVY and PLRV viruses using DAS-ELISA technique.

The calculated data showed that, the occurrence of these viruses were negative in all tested potato plantlets in comparison to the positive control treatment.

Detection of PVX, PVY and PLRV using RT-PCR

The extracted RNA fragments from the resulted plantlets of four tissue-cultured potato cultivars were successfully amplified using cDNAs primed by PVX, PVY and PLRV specific sense and antisense primers as shown in Figures 2, 3 and 4 which showed that the detection of

PVX, PVY and PLRV, respectively by RT-PCR and agarose gel electrophoresis analysis. Data in the illustrated figure (2) declare that, amplicons of expected approximate size for PVX observed only in the positive control (lane 1 at ~750) while, no amplicons appeared in the rested treatments ex. Spunta (lane 2), Cara (lane 3), Selana (lane 4) and Diamond (lane 5) cultivars, respectively.

Fig. 2. Gel electrophoresis analysis of tested potato samples for PVX using RT-PCR utilizing specific PCR primers. Lane L "500-3000bp" DNA Marker, lane (1) positive control infected with PVX (~750bp), Lanes (2-5) potato cultivars (Spunta, Cara, Selana and Diamond) plantlet developed from the shoot tip meristem culture, lane (6) healthy plant control.

While, Data presented in figure (3) showed that amplicons of estimated size for PVY detected only in the positive control (lane 1 at ~801) even though, no amplicons appeared in the rested treatments ex. Spunta (lane 2), Cara (lane 3), Selana (lane 4) and Diamond (lane 5) cultivars, respectively.

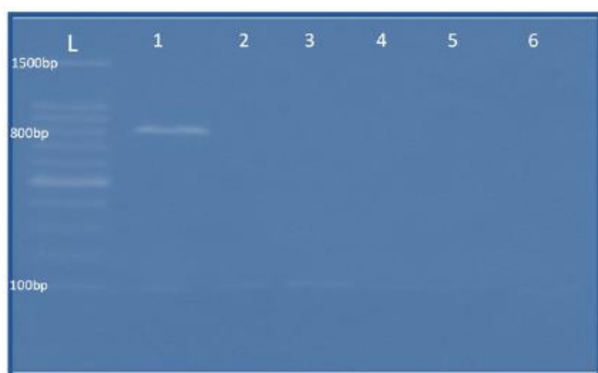

Fig .3. Gel electrophoresis analysis of tested potato samples for PVY using RT-PCR utilizing specific PCR primers. Lane L "100-1500bp" DNA Marker, lane (1) positive control infected with PVY (~801bp), Lanes (2-5) potato cultivars (Spunta, Cara, Selana and Diamond) plantlet developed from the shoot tip meristem culture, lane (6) healthy plant control.

Additionally, figure 4 showed that the amplicons of expected size for PLRV detected only in the positive control (lane 1 at ~548bp) while, no amplicons observed in the rested treatments ex. Spunta (lane 2), Cara (lane 3), Selana (lane 4) and Diamond (lane 5) cultivars, respectively.

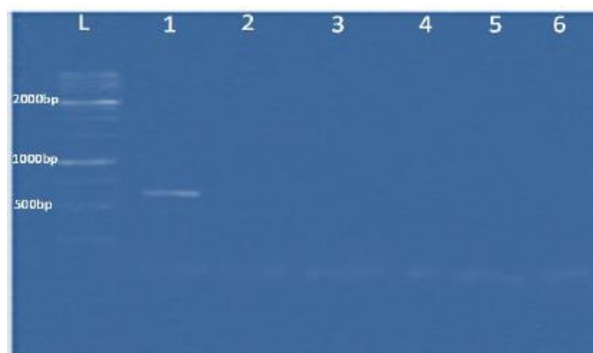

Fig .4. Gel electrophoresis analysis of tested potato samples for PLRV using RT-PCR utilizing specific PCR primers. Lane L "500-2000bp" DNA Marker, lane (1) positive control infected with PLRV (~548bp), Lanes (2-5) potato cultivars (Spunta, Cara, Selana and Diamond) plantlet developed from the shoot tip meristem culture, lane (6) healthy plant control.

Discussion

The obtained results of this study revealed that the medium augmented with ribavirin (50mg/L) decreased the growth of potato shoots length and number of leaves. On the other hand, each shoot tip when it sub cultured on MS basal medium (without plant growth regulators), developed high rate of shoot length, high number of shoots and high number of leaves. Previous results conclude that growth regulators suppress the growth rate of potato plantlets and confirmed that MS control basal medium without growth regulators better than MS medium with growth regulators (Ghaffoor *et al.,* 2003). The interpretation for this result may suggest that potato had enough growth regulators which could help the plantlets to grow faster and better in the MS basal control medium compared to MS medium with growth regulators. So that, adding growth regulators to the medium especially for the plants which have naturally high levels of growth regulators could suppress the growth stages in the plantlets (Abd Elaleem *et al.*, 2009).

Different treatments for potato tubers and shoot tip meristem may enhance virus eradication and increase the possibilities for producing plant free viruses. Thermotherapy treatment with (42°C) for potato tubers and shoot tip meristem enhance the environment for increasing the growth of free virus plant materials and decreased the reproduction stages for viral particles (Fig 2, 3, 4) (Panattoni *et al.,* 2013; Faccioli, 2001). Chemotherapy treatment with ribavirin (50 mg/L) enhanced the environment of the medium for suppressing the reproduction stages for potato viruses PVY, PVX and PLRV (Fig 2, 3, 4). Even though many antiviral chemicals were presented against plant viruses, ribavirin was the most regularly used, according to different types of virus and hosts, along with the virus-host interaction. Previous studies verified that exogenous applications of antivirus representatives repressed synthesis of RNA viruses (Simpkins *et al.*, 1981; Dawson and Lozoya, 1984). Suppression of viral RNA synthesis declined the viral replication machinery and viral particles translocated from the infected cells into the newly divided cells, therefore resulting in production of a greater virus-free area or decreasing viral particles in the treated shoot tips and assisting virus suppression (Singh, 2015). Combination between Thermotherapy and chemotherapy treatments forpotato plantlets enhance the eradication percentages to high percentages of potato viruses compared to single treatments.

Hypothetically, any fragment of a viral genome can be augmented using RT-PCR only if the full length of cDNAs are used, for the reason that the poly (A) is positioned at the 3´ end of the viral genome. But, the genomes of the viruses of interest are usually several thousand bases long, and reverse transcription might be terminated prematurely, resulting in cDNAs of various lengths, starting from the 3` end of the genome. RT-PCR detect the size of the cDNA, as well as identifying fragments that would be most appropriate for detecting tested viruses, indicating the presence of these viral pathogens Thus, the protocol seems appropriate (Puurand *et al*., 1994). The results of RT-PCR showed that the absence of PVX, PVY and PLRV in the resulted plantlets that developed by meristem culture technique and agreed with Zaman, et al. (2001) and Abd Elaleem, et al. (2009).

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تأثير المعاملات الكيميائية والحرارية على تواجد فيروسات البطاطس إكس و واى وإلتفاف الأوراق على البطاطس المنتجة عن طريق زراعة الأنسجة النباتية بإستخدام تقنية القمة النامية

تحسين شعلة [1]، خالد السيد عيد السيد [2] و إبراهيم عبد المنعم الفقى[2]

[1]قسم التكنولوجيا الحيوية البيئية، كلية التكنولوجيا الحيوية، جامعة مصر للعلوم والتكنولوجيا، 6 أكتوبر، جمهورية مصر العربية.

[2]قسم أمراض النبات، كلية الزراعة بمشتهر، جامعة بنها، جمهورية مصر العربية.

يعد محصول البطاطس من أهم المحاصيل على مستوى العالم. تعتبر الأمراض الفيروسية إلتفاف أوراق البطاطس والمرض الفيروسى إكس والمرض الفيروسى واى من أهم الأمراض الفيروسية الشائعة التى تصيب نباتات البطاطس. تستهدف الدراسة الحالية إنتاج نباتات بطاطس خالية من الأمراض الفيروسية عن طريق الماملات الحرارية والكيماوية للقمة النامية لنباتات البطاطس عن طريق زراعة الأنسجة النباتية. أسُتخدمت البراعم النامية على درنات أصناف البطاطس سبونتا وسيلانا و دايموند وكارا كمواد بيولوجية للإختبار عن طريق إستخدام تقنية زراعة الأنسجة النباتية للبراعم النامية .أثناء مراحل الإكثار النباتية تم إستخدم تم إستخدام مادة الريبافيرين المثبطة للفيروسات بتركيز50 ملليجرام لكل لتر من بيئة زراعة الأنسجة مورشاج وسكوج وبملاحظة النتائج ثبت أن أحسن نتائج للنمو الخضرى واالورقى كانت مع معاملات الكنترول بينما ثبطت مادة الريبافيرين بتركيز 50 ملليجرام لكل لتر بيئة نمو النباتات الورقى والخضرى (عدد الأوراق وعدد الأفرع.) بالكشف عن وجود الأمراض الفيروسية إلتفاف الأوراق وفيروسى إكس و واى فى البطاطس بإستخدام داس-إليزا ثبت تثبيط وجود الفيروسات فى القمم النامية المعاملة حراريا وكيماويا مقارنة بالكنترول لكل الأصناف المختبرة.وبإستخدام تقنية تفاعل البلمرة المتسلسل للكشف عن وجود الأمراض الفيروسية إلتفاف الأوراق وإكس و واى فى البطاطس بإستخدام بريمرات متخصصة لكل فيروس ثبت عدم وجود الفيروس فى النباتات الناتجة عن قمة نامية معاملة حراريا وكيماويا مقارنة بوجود الفيروسات فى القمم النامية للعينات الإيجابية وكانت حجم التتابع النيوكليوتيدى للفيروسات الإيجابية النتيجة فى مجرى الجيل رقم (1) حوالى 750 و 801 و 548 تتابع نيوكليوتيدى لفيروسات البطاطس إكس و واى وإلتفاف الأوراق على الترتيب .وبتلخيص كل ما سبق فإن المعاملات الحرارية والكيماوية للقمم النامية لأصناف البطاطس المختلفة تنتج نباتات بطاطس خالية من الأمراض الفيروسية.