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Induced Resistance to Potato Virus Y Potyvirus by Plant Extracts and Salicylic Acid

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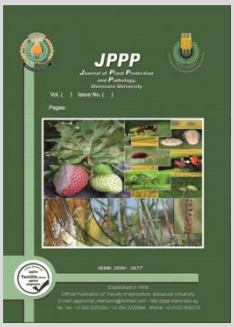

ABSTRACT

The current study was performed to evaluate the potential of two plant extracts (from *Nigella sativa* L. seeds and *Clerodendrum inerme L. leaves)* and salicylic acid to induce systemic resistance (ISR) against *Potato virus Y* (PVY) in potato plants. Ultrastructure investigations using the transmission electron microscope (TEM) exhibited Pinwheels and Ga-Golgi apparatus in the infected plants. Flexuous filaments particles (with a model length of 730×13 nm) were observed in the infected sap using TEM investigation. RT-PCR technique revealed a band- sized at 610 bp in the infected samples with PVY. The infection with PVY was confirmed morphologically and histologically. The most effective treatment for induction of ISR was *Nigella sativa* L., since the highest reduction of virus infection and the lowest inhibition rate (34.7 and 61.7, respectively), were recorded in plants treated with this plant extract and salicylic acid. Peroxidase (POD) activities were 59.7and 37.7 and polyphenol oxidase (PPO) activities were 23.7and 21.0 after 1- and 2-weeks of *Nigella sativa* L. treatment, respectively. On the other hand, salicylic acid had the lowest effect on decreasing PVY infection rate and virus inhibition, resulting in of ISR in potato plants. Taken together, the extracts of *Nigella sativa* and *Clerodendrum inerme* as well as salicylic acid could be used to control PVY infection. A further study is needed to evaluate these materials under field conditions.

Keywords: Potatoes, PVY, ISR, Peroxidase, Histology, TEM.

INTRODUCTION

Potatoes (*Solanum tuberosum*.) are a worldwide economically important member of the family Solanaceae. One of the essential vegetable crops in several countries worldwide is potato. After wheat, maize, and rice, potato is the fourth most important crop economically. It is the first substitute to cereal crops, and it is utilized as a source of starch, carbs, and protein for human consumption as well as for human feed (Horton, 1992). Potato is one of Egypt's most significant and cost-effective vegetable crops. In 2017, the total cultivated area with potatoes was approximately 329721 feddans, yielding around 3659284 tons with an average of 11.098 tons/feddan (Ministry of agriculture and land reclamation economic affairs sector).

Several viruses infect potato plants, producing negative consequences and lowering crop output and tuber quality. There are already about 25 different harmful viruses to potato (Beemstar and Rozendal, 1972). *Potato virus Y* (PVY) is a member of the Potyvirus genus (Family Potyviridae), which is the biggest group of plant viruses, with 111 known and 86 tentative species infecting more than 30 plant families (Fauquet et al., 2005). It has been revealed that PVY strains are closely associated with different degrees of pathogenicity where the most important and common are known to be recombinant (Visser *et al.*, 2012). Primary symptoms of PVY are necrosis or yellowing of leaflets, leaf dropping or sometimes premature death (Eraky *et al.*, 2014 and Abdel-Shafi *et al.*, 2017). In Egypt, PVY was isolated from different crops such as potato, pepper, gladiolus and tomato (Ahmad, 2005 and Al-Nagar, 2007). Also, three isolates of PVY were isolated from naturally infected potato plants cvs. Nicola and Diamont at different locations in Egypt (Mahfouz *et al.*, 2004). Potyvirus virions are rod-shaped flexuous filaments with a length of 680-900 nm and a width of 11-13 nm. The virions contain a monopartite, single-stranded, positive-sense RNA that is approximately 9.7 kb in size. A helical structure of about 2000 subunits of a single coat protein surrounds the viral genomic RNA (Milne, 1988; Shukla *et al.*, 1994; Sabir, 2012)

PVY was detected in potato samples using RT-PCR. The nucleotide and deduced amino acid sequences of the C-terminal portion of the CP gene, as well as the entire 3' UTR, of an Egyptian PVY isolate were analyzed and compared to those of previously reported phylogenic isolates, and secondary structure analysis was used to distinguish between PVY groups (Lorenzen *et al.*, 2006).

Plant extracts have been tested for antiviral activity in a few studies (Chen et al., 2014; Elsharkawy and El-Sawy, 2015 and Mohajer Shojai et al., 2016). The antiviral effect of plant extracts and synthetic compounds is connected to their components, which may act directly on virus particles in the early stages of infection and prevent the release of the virus's nucleic acid, eventually leading to the virus's replication being stopped (Elsharkawy and El-Sawy, 2015 and El-Sawy *et al.*, 2017). Furthermore, these chemicals may function as indirect triggers in plants, inducing systemic resistance to the virus (Al-Ani *et al.*,

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2011; Abdel-Shafi, 2013 and El-Sawy *et al*., 2017). The plant extracts reduced virus concentrations *in vivo*, according to many investigations. Virus replication was suppressed by the extracts of *Sambucus nigra* and *Alium sativum* (Chen et al., 2014 and Mohajer Shojai et al., 2016). Therefore, the objective of this work was to detect and isolate PVY infected potato plants in Egypt as well as to investigate its biological, serological, molecular characterization and the use of safe methods to control this virus.

MATERIALS AND METHODS

Source and inoculum preparation of PVY

Potato virus Y (PVY) was isolated from naturally infected potato (cv. Spunta) plants showing typical symptoms suspected to be a virus, including mild and severe mosaic, vein necrosis, narrow leaf, leaf roll, chlorosis, yellowing, shortening of internodes, mottling and plant stunting. The infected plants were collected from Kafr El-Sheik city, Kafr El-Sheik Governorate, Egypt. The samples were analyzed using particular monoclonal antibodies (mAbs) in an indirect enzyme-linked immunosorbent assay (I-ELISA). against the PVY strain as demonstrated by Koenig (1981).

Single local lesions developed on *Chenopodium amaranticolor* were used to isolate the virus. Mechanical sap inoculation inside an aphid-proof cage under normal glasshouse conditions was used to keep the virus in tobacco (*Nicotiana tabacum* cv. White burley) plants. Phosphate buffer (0.05 M, pH 7.2) (1:1 w: v) was mixed with sap derived from PVY infected tobacco to mechanically inoculate differential hosts. The inoculum of infectious sap was prepared by grinding 5g of the infected potato leaves with 3ml of 0.1 M phosphate buffer, pH 7.2 then filtration through 2 layers of cheese-cloth (Noordam, 1973). Differential hosts used were; Potato (*Solanum tuberosum* cv. Spunta), pepper (*Capsicum annuum* cv. Top star), (*Ch. Amaranticolor, N. glutinosa*) and *N. tabacum* Cv. White burley.

Preparation of plant extracts and salicylic acid

Extracts of Nigella sativa L. seeds and *Clerodendrum inerme* L. leaves were obtained from National Research Center, Giza, Egypt. Twenty-five grams of the fine powder of *N. sativa* L and *C. inerme* were added to 250 mL distilled water and heated in a water bath at 70ºC for 30 min. Three layers of gauze were used to filter the mixture. A cellulose acetate filter (0.022 mm) was used to sterilize the filtrated sap. The solution/suspension was used as a stock with a concentration of 100 % (w/v). Plant extracts at various concentrations (0.5 %, 0.75 %, and 1 % w/v) were mixed with distilled water and tested. While the salicylic acid was used by making aqueous solutions at concentrations of 0.1, 0.2 and 0.3 gm/L. Plant extracts and salicylic acid were used as antiviral agents in the greenhouse and open field. El-Dougdoug *et al.* (2007) explained the method to prepare plant extracts. Inhibitory effects of the plant extracts were evaluated using the following equation (Devi *et al.* 2007):

$$\text{Inhibition \%} = \frac{A-B}{A}$$

Where

A is the number of infected plants in control treatment and B is the number of infected plants in treated plants.

Light and electron microscopy

Epidermal strips taken from PVY-infected potato leaves were used to examine the presence of inclusion bodies (35 days post - inoculation). After staining strips with a mixture of 0.5 % methyl green and pyronine Y, amorphous inclusions were detected using a light microscope (MGP-Y) (Christie and Edwardson, 1977). The following technique for sample preparation was used for the electron microscopic examination, with minor changes, as reported by Luft (1961). Infected leaves were cut into 11 mm pieces and tested. The pieces were fixed in 0.08 M cacodylate buffer pH 7.4 containing 5% glutaraldehyde and 4% paraformaldehyde for 4 h at 4°C. The fixed specimens were washed 3 times with 0.1 M cacodylate buffer pH7.4, containing 3% sucrose, at half-hour intervals. These samples were post-fixed in 1% osmium tetroxide dissolved in a solution of 0.1 M cacodylate buffer (pH 7.4) and 2 % sucrose for 40 min at 4°C. Samples were dehydrated in increasing concentrations of alcohol series, sequentially followed by propylene oxide, then propylene oxide with Epoxy resin (1:1, v: v), and lastly immersed in pure Epoxy resin after 3 h of washing in 0.1 M cacodylate, 3% sucrose (pH 7.4). Thin slices were cut and stained with uranyl acetate and lead citrate before being examined using a JSM1400 plus-JEOL transmission electron microscope (Electronic Microscopy Unit, Faculty of Science, Alexandria University).

Reverse transcription-Polymerase chain reaction (RT-PCR)

Total RNA was extracted from symptomatic potato, *N. tabacum*, pepper and *N. glutinosa* leaves. To amplify the coat protein (CP) gene of PVY, PCR amplification was performed utilizing virus-specific forward and reverse primers (Chikh-Ali *et al*., 2013). According to the manufacturer's instructions, complementary DNA was produced using the MMLV-RT reverse transcriptase enzyme. Primers to amplify CP of PVY included forward PVY: 5′-GATGGTTGCCTTGGATGATG′3 and Reverse PVY: 5′-TAAAAGTAGTACAGGAAAAGCCA as described in Cardin and Moury (2008). The PCR products were run on a 1% agarose gel and visualized with ethidium bromide before being viewed under UV light (Sambrook and Russell 2001).

Evaluation of defense-related enzymes activities

Peroxidase (POD) and polyphenol oxidase (PPO) activities were measured on potato plants treated with plant extracts *Clerodendrum inerme, Nigella sativa*, and salicylic acid after 1 and 2 weeks from viral infection. The upper potato leaves (0.5 g) were collected and frozen in liquid nitrogen before being kept at −80°C. The activity of POD was determined according to the method described by Sudhamoy (2010) while the activity of PPO was measured as described by Flurkey (1989). Three plants were used in each treatment, and the tests were repeated at least 3 times.

Evaluation of plant growth and yield

To determine plant productivity, the number of tubers per plant, weight of marketable tubers, number of marketable tubers/plants, and an average weight of marketable tubers /plant were recorded at harvesting (120 Days from planting).

Statistical analysis

The layout of the experiment was completely randomized. To examine the percentages, they were all converted to arcsine. Using WASP software, data were subjected to statistical analysis and mean calculations. LSD values were calculated at a 5% probability level for mean separations (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

Isolation and identification of the virus

Infected potato plants with mosaic and deformity as the major symptoms were used for virus isolation. PVYO and PVYC specific mAbs produced positive I-ELISA values, whereas PVYN specific mAbs produced negative values. The samples that tested positive caused chlorotic local lesions on *Ch. amaranticolor* (Figure 1 A). The obtained local lesions were used for the inoculation on *Nicotiana tabacum* cv. White burley plants. The isolated PVY strain caused mosaic and vein clearing, which turned into small chlorotic lesions on the tobacco cultivar White burley (Figure 1B). Systemic symptoms such as mosaic and malformation were seen in pepper cv. Top star (Figure 1C) and *Solanum tuberosum* cv. Spunta (Figure 1D, E, F, G).

Due to its importance, PVY is one of the most economically harmful plant viruses. It infects different host species including potato, pepper, *Chenopodium* and tobacco. PVY has a wide range of symptoms and may be spread by insects in a non-persistent manner (Fauquet *et al.*, 2005; Visser *et al.*, 2012). Systemic and local symptoms were induced on potato, *Ch. amranticolor*, *N.tabacum* and pepper (Visser *et al.*, 2012 ; Eraky *et al.*, 2014 ; Abdel-Shafi *et al.*, 2017).

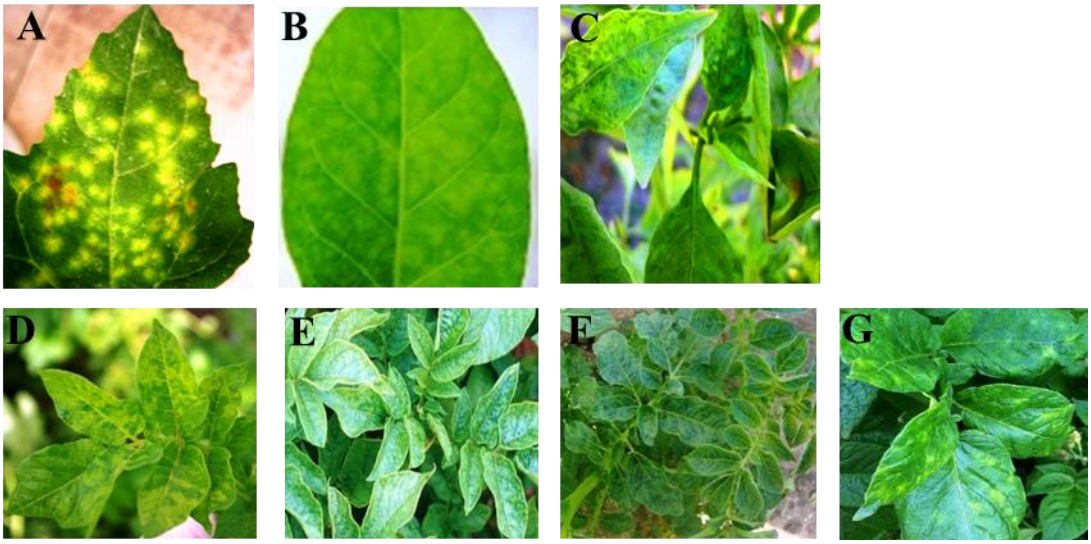

Fig. 1. Host plants inoculated with PVY isolate showing different symptoms such as (A) *Ch. amaranticolor*, (B) *N. tabacum*, (C) pepper and different symptoms on potato plants (D, E, F and G).

PVY-CP gene properties

The total RNA extracted from mechanically inoculated *N. tabaccum* cv. White burly with PVY isolate was evaluated before PCR amplification using spectrophotometer at 260 nm and running on agarose (1.5%). The concentration of total RNA was 3.2 ug per 0.5 gm leaves. The RNA fragment appeared on the agarose gel (total RNA extracted from potato leaves cv. Spounta) indicating the success of total RNA extraction with high concentration (Fig. 2).

The total RNAs from PVY infected with *N. tabacum* L. cv. White burley and potato cv. Spounta leaves were reverse transcribed by RT-PCR using the oligonucleotide downstream primer for PVY-CP gene. The viral cDNA was amplified by PCR using primer sets for PVY/CP-gene. The size of the amplified PCR product of PVY/ CP-gene from RNA of infected potato and *N. tabacuum* leaves were estimated by comparing its electrophoretic mobility with those standard DNA ladder (PGEM DNA marker Promega) as shown in Fig. 2. The amplified DNA was in the expected size of 610 bp., however, with uninfected potato healthy leaves in lane (5), no RT-PCR amplified product was detected.

Multiplex RT-PCR (with specific primers) was utilized to study the isolates belonging to PVY infecting potato exhibiting a band size of 610 bp which is similar to other reports (Lorenzen *et al.*, 2006).

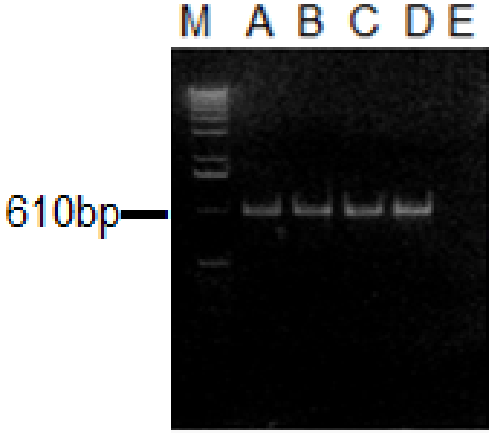

Fig. 2. The integrity of total RNA extraction from mechanically infected potato leaves cv. Spounta and DNA PCR-products of CP gene of PVY isolate using specific primers. M: Marker, A: *N. tabacum* L. cv. white burlym B: Pepper; C: *Ch. amaranticolor*, D: potato plants and E: healthy potato plants.

Light and electron microscopy

Twenty-five days following inoculation, light microscopic examination of infected *N. tabacum* cv. White burley epidermal strip cells showed amorphous inclusion bodies caused by PVY. The inclusion bodies were granular and stained red, and they were found around the nucleus (Figure 3, A). The same infected potato plants (35 days after inoculation) were examined using electron microscope and revealed ultrastructures such as pinwheels (Pw, arrows) in potato mesophyll cells, Ga-Golgi apparatus and M-mitochondria.

Infected tobacco cells produce inclusion bodies, which are visible as amorphous inclusions under a light microscope and as cytoplasmic cylindrical inclusions in the shape of pinwheels, scrolls, and laminated aggregates under an electron microscope (Edwardson *et al.,* 1984; Felczak *et al.,* 2010).

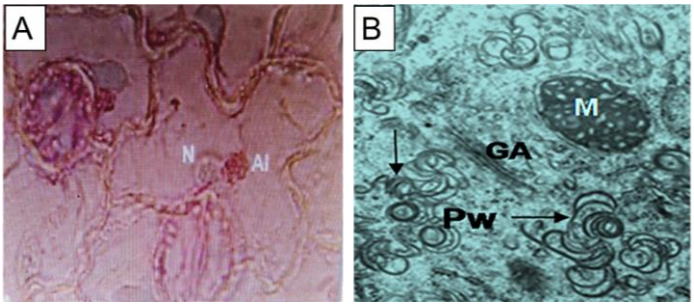

Fig. 3. Amorphous inclusions (AI) stained with red were seen near the nucleus (N) in infected tobacco epidermal strips (A). PVY-induced inclusion bodies in ultrathin sections of infected plants (B), such as *Potato virus Y* cytoplasmic inclusions ultrastructure, Pinwheels (Pw, arrows) in potato mesophyll cells, Ga-Golgi apparatus, M-mitochondria. Magnifications were 400× and 35000×, respectively.

PVY particles morphology

PVY purified preparation from infected potato leaves (negatively stained with uranyl acetate) revealed rod flexible filaments particles with a length of 730×13 nm on transmission electron microscopy (Fig. 4). This observation was confirmed by other researchers (Milne, 1988; Shukla *et al.*, 1994 and Sabir, 2012).

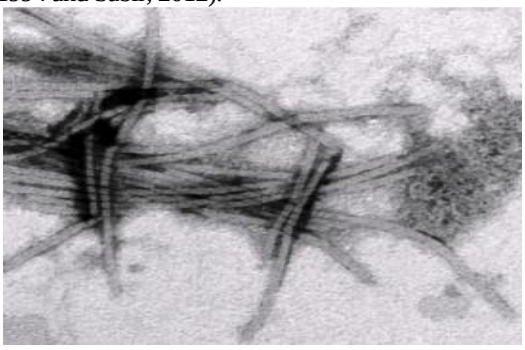

Fig. 4. Electron micrographs of purified negatively stained PVY preparations. Virus particles are flexuous filaments with model length of 730×13 nm. Magnification was set at 40000×.

Induced systemic resistance against PVY

The most effective concentration of plant extracts (1% w/v) was selected for the control of PVY infection. Plant extracts (*N. sativa* L., *C. inerme* L.) and salicylic acid reduced PVY infection in potato plants (44.7%, 34.7%, and 59.2%, respectively) compared with the control (90.7%) (Table 1). This has led to increased rates of virus inhibition (50.7 %, 61.7%, and 34.7%, respectively).

Table 1. Effect of spraying treatments with seed extracts and salicylic acid on *potato virusY* infection.

Treatments	Infection (%)	Inhibition (%)
Clerodendrum inerme	44.7 d	50.7
Nigella sativa	34.7 c	61.7
salicylic acid	59.2 b	34.7
control	90.7 a	-

Using Fisher's LSD at $P \leq 0.05$, different letters indicate significant differences.

Defense-related enzymes

After 1- and 2-weeks post PVY inoculation, the activities of POD and PPO on potato plants were measured. In comparison to control leaves, the activity of POD was substantially enhanced in leaves treated with plant extracts. Similarly, PPO activity was substantially enhanced in treated plants compared with the control (Table 2). Ordinarily, POD and PPO activities in potato plants treated with leaf extracts were higher than in salicylic acid-treated plants.

Commonly, POD and PPO activities were enhanced by the induction treatments. Various mechanisms were activated by antiviral treatments against PVY in potato plants. Treatment of potato plants with extracts (*Nigella sativa* and *Clerodendrum inerme*) and salicylic acid increased the activity of POD and PPO in potato plants. Polymeric polyphenols seem to be more harmful to potential phytopathogens than phenolic monomers (Shukla et al.,1994). Furthermore, the accumulation of antioxidant enzymes (POD, PPO), phenylalanine ammonia - lyase (PAL), and phenolics in chitin-treated banana plants under greenhouse conditions was linked to increased resistance to *Banana bunchy top virus* (BBTV) (Kavino *et al.,* 2008). The function of POD in pathogen resistance was elucidated (Karban.,1989 and Kavino, *et al.,* 2008)

Table 2. The activities of peroxidase and polyphenol oxidase in potato plants after *Clerodendrum inerme, Nigella sativa,* extracts and salicylic acid treatments. Treatments at a concentration of 200 µg mL^{-1} and water as a control were sprayed on potato leaves.

Treatments	Peroxidase		Polyphenol oxidase	
	1WPI	2WPI	1WPI	2WPI
Clerodendrum inerme	49.5 b	39.5 a	27.2 a	21.7 a
Nigella sativa	59.7 a	37.7 a	23.7 b	21.0 a
salicylic acid	34.5 c	27.5 b	15.2 c	13.7 b
Control	21.5 d	18.0 c	10.7 d	9.2 c

Effect of plant extracts and salicylic acid on yield characters of PVY infected potato plants

Nigella sativa and *Clerodendrum inerme* extracts as well as salicylic acid exhibited increase on yield measurements and quality characteristics of potato plants (Table 3). Data showed that the treatments of plant extracts recorded the best results. The control treatment had the lowest values for all of the investigated parameters, whereas salicylic acid had an intermediate value. The extract of *Nigella sativa* achieved the highest results in number of tubers, number of marketable tubers, the average weight of marketable tubers and weight of marketable tubers per plant (9.25, 7.25, 155.07g, and 1.123g, respectively) followed by *Clerodendrum inerm* and salicylic acid, respectively compared with the control (Table 5).

Application of plant extracts (*Nigella sativa* and *Clerodendrum inerme*) and salicylic acid to suppress PVY resulted in a substantial increase in the number of tubers, number of marketable tubers, average weight of marketable tubers and weight of marketable tubers per plant. These results are following other researchers (Sultana *et al*., 2009 and Elsharkawy and El-Sawy, 2015)

Table 3. Effect of plant extracts (*Clerodendrum inerme* and *Nigella sativa*) and salicylic acid on quality and productivity characteristics of potato infected by *potato virusY*

Treatments	No. of tubers/plant	No. of marketable tubers/plant	Average weight of marketable tuber (g)	Weight of marketable tubers (g/plant)
C. inerme	7.75 b	5.50 b	150.07 b	0.825
N. sativa	9.25 a	7.25 a	155.07 a	1.123
salicylic acid	7.25 b	5.25 b	143.85 c	0.756
Control	4.75 c	2.25 c	136.32 d	0.306

CONCLUSION

In conclusion, our results indicated that *Nigella sativa*, *Clerodendrum inerme* and salicylic acid activated POD and PPO, resulting in increased PVY resistance under greenhouse conditions.

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المقاومه المستحثه لفيروس PVY بالمستخلصات النباتيه وحامض السالسيلك

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أجريت هذه الدراسه لتقييم فاعلية نوعين من المستخلصات النباتيه هما حبة البركه *Nigella sativa* و الياسمين البرى (الزفر) *Clerodendrum inerm* و كذلك أستخدام حامض السلسليك salicylic acid فى أستحثاث نباتات البطاطس لمقاومة فيروس PVY . و قد أظهر أستخدام الميكروسكوب الالكترونى (TEM) وجود مشتملات فيروسيه Pinwheels وأجسام جولجى و ظهرت جزيئات الفيروس و كانت عصويه مرنه طولها ٧٣٠X١٣ نانوميتر . أوضحت دراسات RT-PCR أن الوزن الجزئى لفيروس PVY (bp٦١٠). كما اوضحت الدراسات المورفولوجيه و التشريحيه للنباتات المصابه ب PVY أن أفضل المعاملات لاستحثاث المقاومه هو أستخدام مستخلص حبة البركه حيث سجلت أقل نسبة اصابه (٣٤,٧) و أعلى معدل تثبيط للفيرس (٦١,٧) و كان نشاط انزيم البيروكسديز (٥٩,٧ و٧, ٣٧) والبولي فينول اكسديز (٢٣,٧ و٢١) و ذلك بعد مرور ١ و٢ اسبوع من المعامله بالمستخلص على الترتيب و كانت أقل المعاملات أستخدام حامض السلسليك . هناك حاجه إلى مزيد من الدراسه لتقييم هذه المواد في ظل الظروف الحقليه.