Production of Antibodies Specific to an Egyptian Isolate of Potato Virus Y (PVY)

Hatem Elmahdy* and Sayed Abd El Salam

Botany Department, Faculty of science, Cairo University
Cairo, Egypt

*Email: hatemelmahdy [AT] yahoo.com

ABSTRACT---- ***We are reporting here serological tool for detection and identification of a PVY isolate. For the virus isolate detection a number of 3 potato plants (Solanun tuberosum L.) exhibiting rolling, necrotic local lesions and faint mosaic symptoms were collected from three different locations from open fields near Giza city, Egypt in order to detect the presence of PVY, PVX and PLRV for indirect DAS-ELISA test. IgG specific to the viral isolate labeled with alkaline phosphatase was raised. The prepared IgG showed specificity for the virus isolate and there is no serological relationship corresponding to PLRV and PVX.***

Keywords--- **PVY, Detection, ELISA**

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most important crop by production in metric tonnes worldwide, after maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.), and the United States is the fifth largest producer by metric tonnes **(FAO 2012).** Potatoes are the number one vegetable crop in the U.S. **(USDA 2012).** Potatoes are cultivated mainly for fresh consumption, processed foods, production of starch, and for seed tubers.

Potato virus Y (PVY) is the type species of the Potyviridae family and the Potyvirus genus. The Potyvirus genus includes 142 approved and 32 tentative species, making Potyvirus the largest group of plant viruses (**Adams *et al.*, 2011**) and possible members having slightly flexuous filamentous particles having (270 – 900 nm in length and 11 – 15 nm in width) **(Hollings and Brunt, 1981)**. Many countries have reported the presence of potato tuber necrotic ringspots disease (PTNRD) caused by tobacco veinal necrosis strain of potato virus Y (PVY^N) belonged to the sub group tuber necrosis Y (PVY^{NTN}). Potyviruses are transmitted by mechanical inoculation, and most efficiently by aphids in a non-persistant manner, by sticking to aphid mouth parts **(Shukla *et al.*, 1994)**.

(Albrechtsen, 2006) showed serological detection of PVY involves the use of antibodies specific to a strain or a group of strains of the virus. Enzyme-linked immunosorbent assay (ELISA) has been the most common serological technique used in plant virus detection.

Nie XianZhou and Mathuresh Singh (2013) mentioned that the ELISA and RT-PCR analysis revealed that infections with Potato virus S (PVS), Potato virus X (PVX) and PVY, mostly in mixed-infections, occurred commonly in 14 sampled plants. Two strains, namely the common strain (PVY degrees) and the recombinant tuber necrotic strain (PVY^{NTN}) were identified in the PVY-positive plants. In general, mild mosaic was associated with infections with PVX and PVS; intermediated mosaic was associated with PVS and PVY^{NTN} infections; whereas severe leaf deformation/necrosis/drop symptoms were associated with PVY^{NTN} and PVX co-infections, or with PVY degrees and either PVS or PVX co-infections. Virus-free plantlets of potato 'Kennebec' were mechanically inoculated with PVX, PVY degrees , and PVY^{NTN} alone or with PVX+PVY degrees or PVX+PVY^{NTN} combinations in the greenhouse. Single infections with PVY degrees or mixed-infections with PVX+PVY degrees or PVX+PVY^{NTN} incited severe mosaic symptoms and systemic necrosis soon after inoculation; whereas single infections with PVX and PVY^{NTN} induced mild to intermediate mosaic symptoms only.

The objectives of the present study are:

I. Serological identification of PVY isolate.

1- Serological relationship of the viral isolate under test with PVX and PLRV.

2- Preparation of immunoglobulin – G (IgG).

3- Preparation of enzyme conjugated IgG.

4- Determination of IgG-E titer.

5- Specificity of the purified IgG-E.

2. REVIEW OF LITERATURE

Margaritopoulos *et al.* (2009) reported that the serological detection comprises several techniques that can be divided into two groups **(Table 1)**; however Enzyme-linked immunosorbent assay (ELISA), Dot immunoblot assay (DIBA), and Immunosorbent electron microscopy (ISEM) have been the techniques frequently used in the detection of PVY.

Table 1: Groups of serological techniques

Serological detection	
Enzyme-based immunoassays (EIAs)	Other than EIAs
ELISA	Agglutination
DIBA	Gel-diffusion
Tissue blotting immunoassay (TBIA)	Precipitin

Chiunga, E. and Valkonen, J. P. T. (2013) screened from October to December 2011, the occurrence of Potato leaf roll virus (PLRV), Potato virus A (PVA), Potato virus M (PVM), Potato virus S (PVS), Potato virus Y (PVY) and Potato virus X (PVX) was determined in 219 potato plants in 16 fields in the regions of Mbeya (Kawetele, Kikondo, Umalia, Uyole) and Rungwe (Mwakaleli) in Tanzania. Virus-like symptoms observed in most fields included yellowish-green mosaic, leaf rolling, and veinal necrosis. Symptoms in tubers were not studied. Leaves from 10 symptomatic and three symptomless plants were sampled from each field and tested by double antibody sandwich (DAS)-ELISA. PVS and PLRV were detected in 55% and 39% of the samples, respectively, and in all the fields sampled. PVX and PVM were found in most fields, and in 14% and 5% of the samples, respectively. PVA and PVY were only detected in two localities. Co-infection with PVS and PLRV was detected in 14% of the tested plants. Mixed infections involving three or four viruses were detected in 5% of the plants. Twenty samples, which were collected from Uyole and Mwakeleli and found to be ELISA-positive for one or several viruses, were analysed by reverse-transcription PCR using virus-specific primers designed to amplify the coat protein (CP) encoding region. All ELISA-positive samples tested positive by reverse transcriptase (RT)-PCR. Four and five samples ELISA-negative for PVX or PVA, respectively, were positive when tested by RT-PCR, suggesting that the actual incidence of these viruses may be higher than detected by DAS-ELISA. The PCR products from 3-5 samples per virus were sequenced without cloning, which reconfirmed detection of PLRV, PVA, PVS, PVX and PVM. This is thought to be the first report of the five viruses infecting potato in Tanzania.

Hutton, F. *et al.* (2013) mentioned that potato tuber necrotic ringspot disease (PTNRD) as described by Kus et al., (1992) is found worldwide but has not previously been identified in Ireland. In recent years necrotic symptoms, veinal necrosis, spots, mottling, mosaics and chlorosis have been observed on potato foliage in different regions across Ireland. In 2011 and 2012, affected leaves were serologically tested by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA). In some instances, these symptoms were associated with PVY. Further analysis of PVY positive samples using RT-PCR confirmed the presence of the recombinant strains PVY^{NTN} and PVY^{N:O}. No tuber samples were available to determine if their presence was associated with PNTRD. In 2013, foliage of the cultivar Nicola grown in County Carlow, Ireland, showing mosaic, veinal necrosis and chlorosis was sampled and serologically and molecularly analysed. Presence of PVY was confirmed by DAS-ELISA, and RT-PCR analysis confirmed the presence of both PVY^{NTN} and PVY^{N:O}. Tubers from the infected

plants were harvested and, following storage at ambient temperature for 28 days, developed the typical superficial necrotic ringspot disease. This is thought to be the first report of PTNRD associated with PVY recombinant strains in Ireland.

Syller, J. and Grupa, A. (2014) described the influence of co-infection on concentration and accumulation of genetically different isolates of Potato virus Y (PVY) in potato and tobacco plants and the efficiency of transmission by Myzus persicae of PVY isolates from doubly versus singly infected plants were evaluated. The vector ability to simultaneously transmit two virus isolates was examined. Eight PVY isolates represented three strain groups: PVY^O (pathotype and serotype O), PVY^NW (pathotype N and serotype O), and PVY^{NTN} (pathotype and serotype N). Different diagnostic methods, including: DAS-ELISA, aphid transmission tests and bioassays, were applied to detect the presence of PVY isolates in source and assay plants.

★★ Antigenic characterization:

(Hollings and Brunt, 1981) showed the serological relationships among definitive potyviruses are complex and range from very closed to intermediate to not detectable. Although most of the genus members are serologically related to at least one other member in the group and in many cases to several others, the expected serological relationships between many connected pairs have not been observed. For example, bean yellow mosaic potyvirus (BYMV) is serologically related to lettuce mosaic potyvirus (LMV) and bean common mosaic potyvirus (BCMV), but no serological relationship has been observed between LMV and BCMV **(Hollings and Brunt, 1981** and **Francki et al., 1991).**

Harrison (1985) has been suggested that there is no simple pattern of antigenic relationships often does correlate with biological properties. In this respect potyviruses are perhaps exceptions to the rule that viruses which are antigenically related also share most of their other properties.

(Murphy *et al.*, 1995) reported the viral proteins of potyviruses are moderately immunogenic, there are serological relationships between members. A conserved internal, trypsin-resistant core coat protein (CP) epitope has been identified, which is, shared by most members of the family. They also showed those one monoclonal antibody (MAb) reacts with most aphid-transmitted potyviruses. The CP amino acids sequence homology among aphid transmitted viruses is 40-70% Some species are serologically related to species in the genera *Reymovirus* and *Bymovirus*.

Liave *et al.*, (1999) established a serological relationship of PVY isolates belonging to the pepper pathotypes 0, I and 1-2 By ELISA. They did not react with MAbs, which typically recognize non-pepper strains within the PVY group, leading to discrimination between these two groups of strains. No serological differences were found between the three PVY pepper pathotypes.

Varveri (2000) produced antiserum against PVY for detecting a concentration of 10 ng/ml-purified virus by double antibody sandwich (DAS)-ELISA technique.

3. MATERIALS AND METHODS

This investigation was carried out at AGERI, ARC, Giza, Egypt.

A number of 3 potato plants (*Solanun tuberosum* L.) exhibiting rolling, necrotic local lesions and faint mosaic symptoms were collected from three different locations from open fields near Giza city, Egypt in order to detect the presence of PVY, PVX and PLRV for indirect DAS-ELISA test.

They were transplanted at AGERI greenhouse under a controlled temp. (24^{o}-30^{o}C) and the following tests were carried out:

1. Serological identification of the PVY isolate.

1.1. Serological relationship of the viral isolate under test with PVX and PVY.

For determination of the serologiocal relationship between the viral isolate under test and both PVX and PLRV, indirect-enzyme linked immunosorbent assay (I-ELISA) technique was used as described by **Clark and Adams (1977)**. 200 µ1 of sap from virus (viral isolate under test, PVX and PVY)- infected leaf plants propared in coating buffer, pH 9.6 (1:10, w:v) were separately added to the microtiter ELISA plate. After incubation at 37^{o}C for 3 h., the plate was washed three times (5 min. for each) using waching buffer (PBS- Tween 20, PBST). 400 µ1 of 3% bovine serum albumin (BSA)

were added for each well and the plate was incubated for 1 h. at 37°C. After waching, 200 µ1 of each of IgG specific to PVX and PVY obtained from AGERI were added (two wells for each virus as replicates were used). After incubation for 4 h. at 37°C, the plate was washed and then 200 µ1 of the Universal IgG-E with a dilution of 1: 3000 obtained from AGERI were added to each well followed by incubation for 3 h. at 37°C. After washing, 200 µ1 of the substrate with a concentration of 0.5mg/ ml substrate buffer were added. The ELISA values at 405 nm were determined 5, 10, 15 and 20 min. post incubation at 37°C. As control, a healthy sap from the same plants was used (two wells for each plant).It is important to mention that 200 µ1 of coating buffer were added to the well 1A instead of the sample to serve as blank. The ELISA value over the two folds of the healthy ones was considered as a positive result.

1.2. Preparation of PVY antiserum.

The antiserum of PVY was raised based on the method of **Clerk and Adams (1977).** Two New Zealand white rabbits (5Kg) were immunized by intramuscular injection 0.5 ml of purified virus (1 mg/ml), and equal volume of Freund's complete adjuvant. Three intramuscular injections with 7 days interval three weeks post the first injection were carried out. For each, 0.5 ml (1 mg/ml)of purified virus and equal volume of Freund's incomplete adjuvant were used . The rabbit was bled after 10 days of last injection. The blood was collected and incubated at 37 °C for 1 h followed by centrifugation at 14000 rpm in GSA rotor at RT. The antiserum was then collected and stored at 4 °C for further studies.

1.3. Purifiction of immunoglobulin - G (IgG).

The IgG was purified according to the method described by **Clark and Adams (1977).**

★★ Procedure:

One ml of the crude antiserum was diluted with 9 ml d.H_2O and poured into 30 ml centrifuge tube, followed by dropwise addition of 10 ml saturated ammonium sulphate, with stirring, the mixture was then kept at RT for 1 hr. the protein was precipitated by centrifugation at 8000 rpm in the JA 20 rotor for 15 min at 10°C. The supernatant was discarded and the pellet was resuspended in 1 ml half – strength PBS, pH 7.4 .The protein suspension was dialyzed three times against 1L of half – strength PBS and twice , for about two hr at RT and overnight at 4 °C. Dialyzed protein was loaded on 10 ml column of DEAE cellulose suspension, and eluted using half - strength PBS, and 1 ml was collected per tube. The absorption was measured for each fraction at 280 nm and IgG concentration was adjusted to 1 mg/ml (A_{280} nm = 1.4) in half - strength PBS and stored at 4 °C. In order to minimize background in serological assays, 2 ml of crude antiserum were cross- absorbed by mixing it with 8 ml of crude sap from healthy leaves of *D.metel*.

1.4. Preparation of enzyme conjugated IgG.

On the basis of the method given by **Clark and Adams (1977)** and **Abdel- halim et al. (2000),** the enzyme conjugated IgG was prepared with some modifications.

★★ Procedure:

Alkaline phosphatase (AP) was dialyzed first in 100 ml of 0.06% glutaraldhyde in full strength PBS for 1 h. then 1 mg IgG was added to the AP and dialyzed for another hour in the same solution. This mixture was dialyzed three times against full strength PBS, twice for about 2 h. at RT and once for overnight at 4 °C. The enzyme conjugated IgG (IgG-E) was recovered from the dialysis tubes, and transferred to a glass vial. BSA (5mg/ml) was added to increase the stability of the conjugate that was then stored at 4 °C until use.

1.5. Determination of IgG-E titer.

To determine the concentration of the conjugate (IgG-E), 10^{-1} dilution was prepared by adding 100 µl IgG-E to 900 µl d. H_2O followed by spectrophotometering at wave length 280 nm. The concentration of IgG- E was calculated as recommended by **Clerk and Adams (1977)** reported that 1.46 O.D. equal 1 mg/ml protein.

1.6. Specificty of the purified IgG-E.

In this experiment, double antibody sandwich - ELISA (DAS- ELISA) technique was applied using saps extracted from PLRV-, PVX- infected potato leaf and PVY- infected *D.metel* leaf (dilution 1/5 for each), as well as purified virus preparation (10^{-1}) Saps extracted from healthy (dilution 1/5) either *D.metel* or potato plant leaves were also used as control . 200 µl of produced - IgG (1 mg/ml) in coating buffer, pH 9.6 (1 µl /ml buffer) were added to each well in the

ELISA plate. After incubation at 37°C for 3 h. the plate was washed three times (5 min for each) using PBST. 200 µl of each sap in sample buffer were added, two wells were used as replicated for each sap. To the well 1A, 200 µl of the sample buffer were added to serve as blank. The plate was incubated for 3 h. at 37 °C followed by washing as mentioned before. Again 200 µl of PVY-IgG (1 mg/ml) in conjugate buffer, pH7.4 (1 µl / ml buffer) were added to each well in the ELISA plate. After incubation at 37 °C for 3 h. the plate was washed three times (5 min for each) using PBST. 200 µl of freshly prepared substrate buffer containing ρ- nitrophenyl phosphate (ρ- NPP) with concentration of 0.5 mg per ml buffer were added. The ELISA values at 405 nm were determined after 2 h. post incubation at 37 °C. The sample that gave an ELISA value average two folds over that belonging to the healthy sample was considered as positive result.

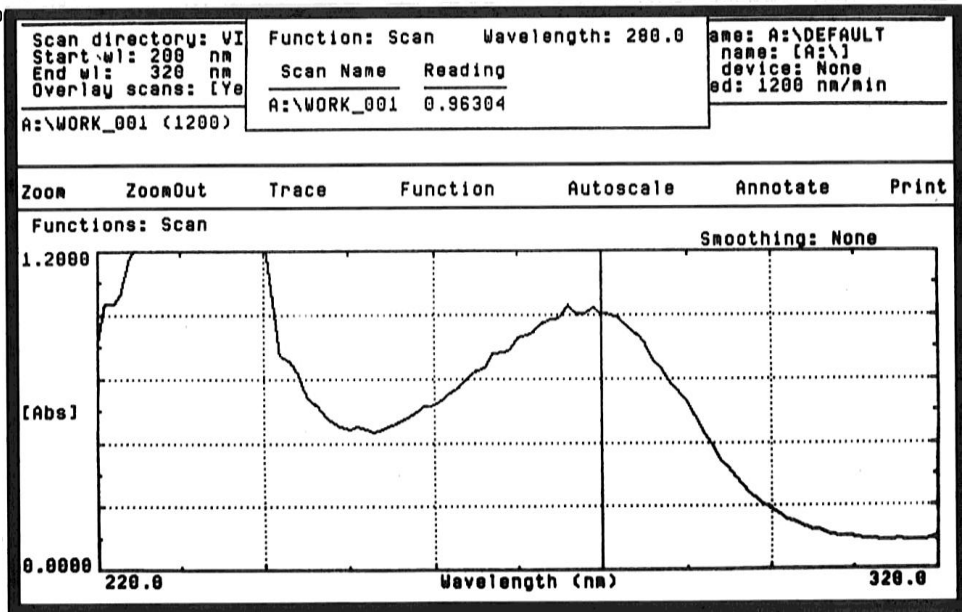
4. RESULTS

1. Serological characterization of PVY isolate.

1.1. Raising antiserum specific to the virus isolate.

A specific antiserum (IgG) to the virus isolate was produced, purified and suspended in a volume of 1 ml followed by determination of its titre by measuring the absorbance of the diluted preparation (10^{-1}) at 280 nm .

Based on the formula (1.46 O.D. at 280 nm represent 1 mg protein/ ml) data in **(Figure 1)**, show that the A_{280} of diluted IgG was 0.963 and the total yield of purified IgG specific to the virus isolate was 6.596 mg. for further studies the co



2.1. Specificity of the produced IgG.

The indirect DAS-ELISA technique was used to test the specificity of the purified IgG to detect the virus isolate under test in purified virus preparation as well as virus – infected *S. tuberosum* plants.

Data in **table (2)** show a good specificity of the obtained IgG to the virus isolate under test. There is serological relationship between PLRV or PVX and the virus isolate under test .

Table (2): specificity of the produced IgG using indirect DAS- ELISA

Sample	ELISA detection	
	ELISA value	Result
Purified virus.	1.943	+
PLRV- infected sample.	0.263	-
PVX- infected sample.	0.248	-
Virus – infected ***S. tuberosum.***	1.373	+
Healthy ***S. tuberosum.***	0.287	-

+ : Positive - : Negative

5. DISCUSSION

Potato virus Y (PVY, Potyvirus) is the fifth most important plant virus worldwide in terms of economic and scientific impact. It infects members of the family Solanaceae and causes losses in potato, tomato, tobacco, pepper and

petunia production **Tomczynska, I. *et al*., (2014)**. PVY transmitted by a variety of aphid species in a non-persistent manner and through plant sap. **Kamangar, S. B. et al., (2014)**. **Takas (1999)** reported that PVY is a member of the potyvirus group in the potyviridae family.

In this investigation serological studies using PAbs with indirect DAS-ELISA technique described by **Koeing and Paul (1982)** was carried out for PLRV, PVX and PVY detection in potato samples under investigation using the specific antisera for PLRV, PVX and PVY viruses. This is in consistence with **Garcia and Garcia (2000)** who showed that total PVY was serologically determined using PAbs with the DAS-ELISA. Also, **Pooja Sharma *et al*., (2013)** and **Al-Saikhan, M. S. *et al*., (2014)** evaluated and showed the positive result against antiserum specific for PVY and the use of ELISA technique for rapid detection of PVY in potato plants.

The viral proteins of potyviruses are moderately immunogenic, there are serological relationships between members **(Murphy *et al*., 1995)**. Both polyclonal **(Abdel-Halim *et al*., 2000)** and monoclonal **(Liave *et al*., 1999 and Liu *et al*., 1999)** antibodies were produced against some potyviruses. In this work, a specific antiserum raised against our viral isolate under test followed by purification of the IgG and labeling with alkaline phosphatase for virus detection using DAS-ELISA technique. The application of this antiserum showed specificity to our isolate. In addition no serological relationship corresponding to PLRV and PVX was found. The serological relationships among definitive potyviruses are complex and range from closed to intermediate to not detectable **(Hollings and Brunt, 1981). Gugerli and Fries (1983) and Jordan and Hammond (1991)** reported that most PVY strains or pathotypes are serologically closely related. Therefore, methods based on the antigenic properties have only occasionally resulted in successful discrimination between them.

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