Optimum Conditions for Detecting Potato Virus Y (PVY) by Enzyme Linked Immunosorbent Assay (ELISA) Using Locally Produced Antiserum

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ABSTRACT

Potato Virus Y (PVY) is a destructive Virus of Potato (*Solanum tuberosum* L.).Commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits are used to index PVY. A commercially available kit for five hundred tests costs Rs. 60,000 to 70,000. Therefore local production of an antiserum for the virus saves national expenditure. The antiserum for PVY produced locally, was used through out the study. Antibodies were raised by injecting 0.26 mg of the purified PVY mixed with 0.5 cubic centimeters (cc) of Sodium and potassium phosphate buffer which was diluted in 1.0 cc of 0.85 percent sodium chloride, to a Rabbit intraveinally. One week after the first, 0.39 mg of the virus was mixed with 0.25 cc of 0.01 M Sodium and potassium phosphate buffer, mixed with 1.0 cc of incomplete freund's adjuvant and injected intramuscularly. The rest of the three injections containing 0.52, 0.65 and 0.78 mg of the virus was mixed with 1.00 cc of each incomplete freund's adjuvant respectively and injected intramuscularly at one week intervals. Bleeding of the Rabbit was done, after one week of the second injection and the final injection. Blood was incubated at room temperature for two hours and kept at 10°C overnight. The serum was separated using a sterile pasteur pipette and processed by adding an equal volume of glycerol and sodium azide in which the final concentration of sodium azide was adjusted to 0.025 percent. Antiserum was absorbed with direct healthy Potato sap for one method and with partially purified healthy Potato sap for the other method.

Indirect ELISA was performed for optimization of a suitable extraction buffer, suitable bleed, method of absorption, Dilution of the antiserum and incubation period after adding p-nitrophenyl phosphate substrate. Best extraction buffer was 0.5 M Potassium phosphate + 0.1 M Sodium EDTA + 1 percent sodium sulphite (pH 7.5). Best antiserum was obtained from the first bleed after absorbing with partially purified healthy Potato sap. Most suitable dilution of the antiserum was selected as 1:400 to 1:600 in PBS-TPO buffer. The conjugate dilution was maintained as 1:2000 in PBS-TPO buffer through out the study. The study revealed the locally produced antiserum is suitable for PVY indexing successfully.

KEY WORDS: Enzyme Linked Immunosorbent Assay (ELISA), Phosphate Buffered Saline +Tween 20 + Polyvinylpyrrolidone + Ovalbumin (PBS-TPO), Potato Virus Y (PVY).

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the second most used food crop (Vincent Marti'nez C.,), in the world with annual production of 300 million tons (Anon, 2002). It is the staple food of some European countries and also consumed as a vegetable with the main diet. In Sri Lanka Potato is grown in large extents in Badulla (4064 hectares in 2005) and Nuwara Eliya (1291 hectares in 2005) districts. Minor extent cultivations are found in Jaffna, Puttalam, Vavuniya, Anuradhapura, Kandy, Matale, Matara and Ratnapura districts. All the areas under Potato cultivation in Sri Lanka have accounted for 76919 tons of national production in year 2005, including 56295 tons from Badulla and 20004 tons from Nuwara Eliya districts (Anon, 2005).

Potato Virus Y (PVY) which is a filamentous, flexuous, non-enveloped RNA virus (730 nm in length, 11 nm in width, 3.3 nm from the pitch) is the second most important virus of Potato. It accounts for 9,704 nucleotide genome size, 5.4-6.4 percent RNA (Leiser and Ritcher, 1978-CD ROM), 93.6-94.6 percent proteins in virion particles. PVY, which is called as vein banding mosaic or severe mosaic, can cause severe damage to the crop leading 10-100 percent yield loss (de Bokx and Huttinga, 1981:Salazar, 1996-CD ROM). PVY infected plants show severe to no symptoms. When symptoms are present plants show very mild to severe yellow mottling, stunted growth, rugose appearance, stunting and crinkling. PVY is transmitted through infected tubers and aphids in non-persistent manner.

Enzyme Linked Immunosorbent Assay (ELISA) is widely applied to detect PVY. One of the commercially available ELISA kits (DAS ELISA) used to detect PVY is around 60,000 to 70,000 in Sri Lankan Rupees. Using a single ELISA kit only five hundred tests can be performed. Since Srilanka is a third world country importing ELISA kits is not economically feasible. Therefore production of a local antiserum is the alternative to perform ELISA with minimum expenditure.

MATERIALS AND METHODS

The Research was carried out at Plant Virus Indexing Centre, Gabadawatta, Homagama, Sri Lanka from January 2006 to July 2006. The antiserum was prepared at, Animal unit, Medical Research Institute, Borella, Colombo-08, Sri Lanka.

Potato samples were collected from Agriculture Research station, Sita Eliya, Nuwara Eliya, Sri Lanka and from farmer fields, Sprouted Potato tubers planted at Plant Virus Indexing Centre. Leaves from the grown up plants, tissue cultured plantlets and

positive and negative kit controls were used for ELISA.

Inoculation of Nicotiana Glutinosa

Nicotiana glutinosa, which is a host species of PVY, was inoculated to obtain PVY positive samples to perform ELISA. PVY infected Potato tissue (0.1g) was ground with 0.1 M Potassium phosphate buffer and 0.01 percent Sodium ethylenediaminetetraacetate (Sodium-EDTA). The 400-mesh carborundum powder was used as the abrasive, the sap was applied on the surface of the third leaf, from petiole end towards the tip. The inoculated plant was covered with moist papers for overnight.

Virus Purification

One hundred grams (100g) of PVY infected Potato leaf material was homogenized with 2 volumes (w/v) of 0.2 M Sodium and potassium phosphate buffer (pH 8.0), with 0.2 percent 2-mercaptoethanol and 0.01 M of Sodium-EDTA. The homogenate was filtered twice through three folds of gauze. The filtrate was centrifuged at 7500 r.p.m for 20 minutes. To the supernatant, one percent of Triton X-100 was added and stirred at 4°C for two hours and centrifuged at 7500 r.p.m for 20 minutes. The supernatant was separated and 0.2 M Sodium chloride and four percent Poly ethylene glycol (Molecular weight of 8000) was added and stirred at 4°C for one hour. The mixture was incubated at room temperature for another one hour and centrifuged at 7500 r.p.m for 20 minutes. To the pellet 0.02 M Sodium and potassium phosphate buffer (pH 7.2), 1 percent Triton-X 100 was added ($1/5^{th}$ of the original volume) and kept at 4°C for overnight. The mixture was stirred at 4°C for 30 minutes and centrifuged at 6000 r.p.m for ten minutes. The supernatant was separated and centrifuged on a 30 percent sucrose cushion at 26000 r.p.m (SORVALL ULTRA 80 centrifuge/Rotor No. A-641) for two and half hours. The pellet was obtained as partially purified PVY (With minor modifications to the CIP purification protocol-Anon, 1999b).

Determination of the Concentration of the Virus

Twenty micro liters of the virus pellet was diluted 1:50 (v/v) in 0.01 M Sodium and potassium phosphate buffer (pH 7.2) and Optical density (OD) was measured at 260 nm and 280 nm respectively using the UV spectrophotometer. OD_{280}/OD_{260} ratio was standardized with the virus extinction coefficient of PVY *i.e* 2.3.

Immunization of the Rabbit

Antibodies were raised by injecting 0.26 mg of the purified virus mixed with 0.5 cubic centimeters (cc) of the 0.01 M Sodium and potassium buffer diluted in 1.00 cc of 0.85 percent Sodium chloride to the marginal ear vein of a New Zealand white Rabbit which is six months in age and 3.5 Kg in weight, as the first injection (Intrveinal). The second injection (First intramuscular injection) with 0.39 mg of the virus was mixed with 1.00 cc of incomplete Freund's adjuvant and injected to a rear leg of the rabbit. Third, fourth and the fifth injections were given with 0.52, 0.65, 0.78 mg of the virus mixed with 1.00 cc of incomplete Freund's adjuvant respectively.

Antiserum Production

Bleeding of the rabbit was done at one week after the second injection and a week after the final injection. The blood was incubated at room temperature for two hours and kept at 10°C overnight. The serum was separated using a sterile pasteur pipette and centrifuged at 5000 r.p.m for 10 minutes and mixed with an equal volume of glycerol. To the antiserum 12.5 percent Sodium azide was added adjusting the final concentration of the Sodium azide to 0.025 percent in order to preserve the antiserum from microorganisms. Antisera were stored at -20°C.

Absorption of Non-specific Antibodies in Antisera

Absorption of the antisera was done in two methods. In the first method the non-specific antibodies were absorbed with the healthy direct Potato sap, and the second method the antiserum was absorbed with partially purified healthy Potato sap.

Obtaining Direct Healthy Potato sap for method one

Healthy Potato leaf tissues were crushed and filtered through a muslin cloth and obtained the sap.

Obtaining Partially Purified Healthy Potato Sap for Method Two

Healthy Potato tissues were undergone the PVY purification procedure omitting addition of 30 percent sucrose. The pellet obtained after centrifugation (26000 r.p.m for two and half hours) was used as partially purified healthy Potato sap.

Absorption

One milliliter of the antiserum was mixed with equal volume of the direct Potato sap or partially purified healthy Potato sap. The mixture was kept still for four hours at room temperature and centrifuged (IEC Centra-4B/Rotor No. CAT 837) at 11000 r.p.m for 10 minutes. The supernatant was collected as the absorbed antiserum.

Performing ELISA

Leaf samples were homogenized 1:10 (w/v) with the extraction buffer and centrifuged at 6000 r.p.m for five minutes. The supernatants were pipetted in to the wells of the ELISA plate with 200 µl per well and two replicates per sample. The plate was kept in a moist box and kept at 4°C overnight.

The plate was washed with PBS-T (Two quick washes and three prolong washes at five minute intervals) and the wells were filled with the antiserum diluted in PBS-TPO, 200 µl per well. The plate was incubated in the moist box at 37°C for 2 hours.

The plate was washed with PBS-T and the wells were filled with the Protein A conjugate diluted in PBS-TPO, 200 µl per well. The plate was kept in the moist box and incubated at 37°C for two hours.

The plate was washed with PBS-T and p-nitro phenyl substrate diluted (50mg tablet/ 5ml) in the substrate buffer (p-nitro phenyl phosphate substrate buffer-pH9.8) was added, 200 µl per well. The plate was kept in the moist box and incubated at 37°C for two hours. Absorbance values were taken at 405 nm using the ELISA reader (anthos 2020/ version 1.4).

Optimization of Conditions to Detect by ELISA

Optimization of Sample Extraction Buffer

Two extraction buffers: 0.5M potassium phosphate+0.1 M EDTA+1 percent sodium sulphite (pH 7.5) and PBS-T+2 percent polyvinyl pyrrolidone (PBS-T+2% PVP-pH 7.4) were tested for sample extraction to find out the best.

Optimization of Different Antisera

Absorbed (with healthy direct sap) and non-absorbed antisera from the second bleed, absorbed (with healthy direct sap) antisera from first and second bleed, antisera from the first bleed absorbed with direct healthy sap and partially purified healthy sap were compared.

Optimization of Different Dilutions

The antiserum selected from the former tests was used to find the best dilution. The antiserum was diluted in PBS-TPO in 1:200, 1:400, 1:600 and 1:800 dilutions.

Optimization of Different Substrate Incubation Periods at 37°C

Absorbance values for 405 nm were taken at 30 minutes, 1 hour, 1 ½, 2, 2 ½, hours time periods.

Conjugate Dilution

The dilution of the Protein A conjugate was maintained at 1:2000 in PBS-TPO through out the study.

Table 1- Positive and Negative Samples:

Negative		Positive	
No.	**Name.**	**No.**	**Name.**
H_K	Agdia	D_K	Agdia
H_1	T/C-20	D_1	1-2
H_2	D (D) T3-2	D_2	1-6
H_3	SE(-)	D_3	G (E) T5-6-2 (Da)
H_4	TRI-2	D_4	SE (+) 1
H_5	TRI-3	D_5	SE (+) 2
H_6	Sprout-10	D_6	Sprout 1
H_7	GLBW		

Identification of Positive and Negative Samples

Positive and negative controls used for testing the locally produced antisera were confirmed by commercially available DAS ELISA kits. Average buffer values at 405 nm were subtracted from average disease and healthy values to obtain disease and healthy values. Threshold value (THV) was calculated by multiplying the healthy value by two. The sample values greater or lesser than THV were identified as Positive or negative samples respectively.

Interpretation of ELISA Data

For getting optimum conditions the ratio of disease and healthy values were considered. Therefore in comparison of effectiveness of the antisera, best dilution and optimum incubation period was selected by observing disease / healthy ratio.

RESULTS AND DISCUSSION

Virus Purification

Virus yield obtained from the first purification was 1.04 mg. During the second purification it was 1.56 mg .Total Virus yield was 2.6 mg.

Buffer Comparison

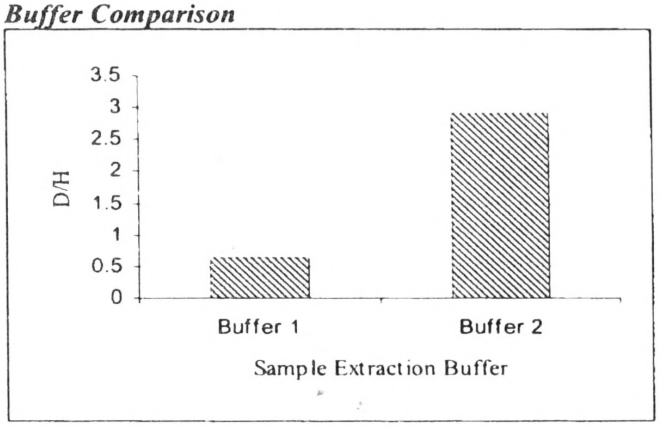


Figure 1-Comparison of different Extraction buffers:

$D_{ave.} = D_1$, D_2: $H_{ave.} = H_1$

Buffer 1 = PBST+2% PVP (pH 7.4)
Buffer 2= 0.5M potassium phosphate + 0. 1 M Sodium-EDTA + 1% Sodium sulphite (pH 7.5)

Buffer 2 gave highest disease / healthy ratio. Therefore 0.5 M Potassium phosphate + 0.1 M Sodium EDTA + 1 percent Sodium sulphite (pH 7.5) was selected for further extraction purposes.

Comparison of Absorbed and Non-absorbed Antisera from the Second Bleed

$D_{ave.} = D_3$, $H_{ave.} = H_2$: *N.glutinosa* positive and negative controls

Absorbed form of the second antiserum resulted higher disease / healthy ratio for both Potato and *N.glutinosa*.

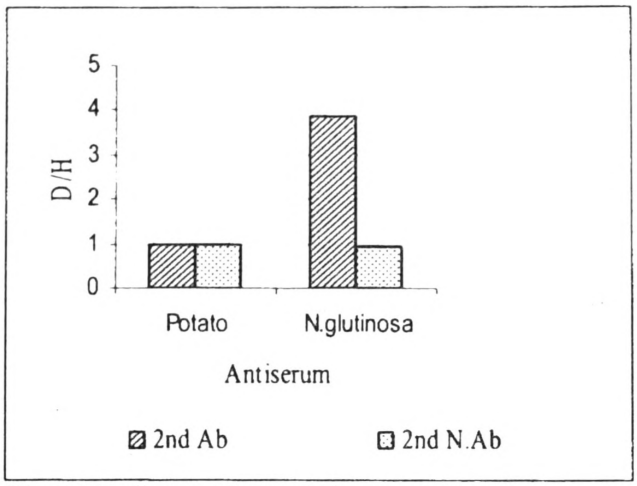


Figure 2 -Comparison of absorbed and non-absorbed antisera of the second bleed:

Comparison of absorbed antiserum from the first bleed and the absorbed from the second bleed (Absorbed with direct healthy sap)

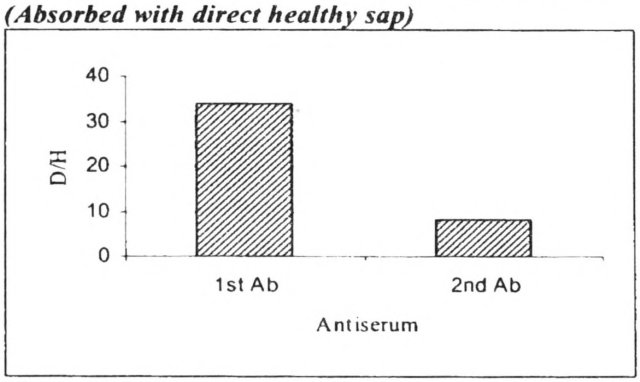


Figure 3-Comparison of absorbed antisera from the first bleed and second bleed (Absorbed with direct healthy sap):

$D_{ave.} = D_4, D_5$: $H_{ave.} = H_3$

Absorbed antiserum from the first bleed gave highest disease to healthy ratio. Therefore the first bleed was identified as the best.

Comparison of absorbed antisera from the first bleed absorbed with direct healthy sap and partially purified healthy sap

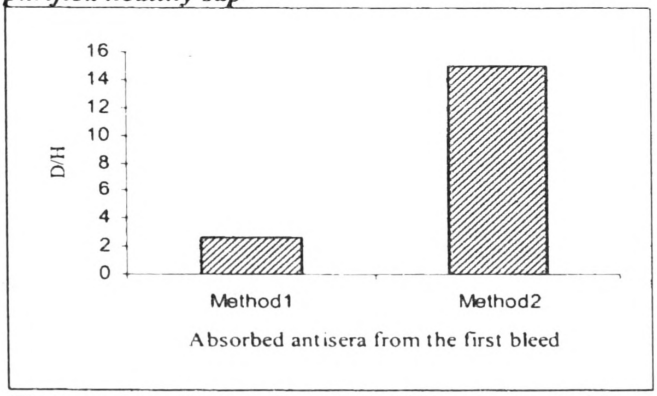
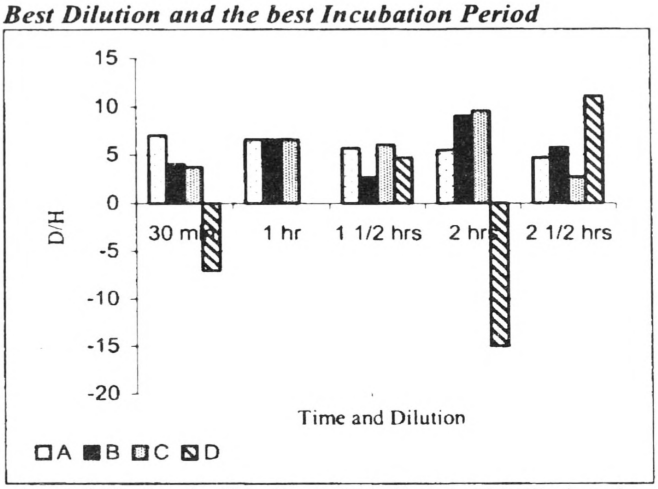


Figure 4 - Comparison of different absorbing methods:

$D_{ave} = D_6$: $H_{ave} = H_4, H_5, H_6$

Absorbed antiserum from the first bleed that was absorbed with partially purified healthy sap gave highest disease/healthy ratio. Therefore the absorbed antiserum from the first bleed absorbed with partially purified healthy sap was selected to check for the optimum incubation period and the dilution.

Best Dilution and the best Incubation Period



A = 1:200, B = 1: 400, C = 1:600, D = 1: 800 dilutions in PBS – TPO.

Figure 5- Best dilution of the antiserum from the first bleed absorbed with partially purified healthy sap and the incubation period after adding the p- nitrophenyl phosphate substrate:

$D_{ave.} = D_4, D6$: $H_{ave.} = H_6, H_7$

Dilution 1:800 gave the highest disease / healthy ratio at 2 ½ hours. But a negative disease / healthy ratio was resulted by 1:800 dilution at 30 minutes and 2 hour incubation period due to high buffer reactions which is not a suitable condition for ELISA. The best two disease / healthy ratios were obtained at 2 hours of incubation period for 1:400 and 1:600 dilution levels. Therefore 1:400 to 1:600 dilutions of the antiserum and two hour incubation period is ideal to detect PVY using ELISA.

Reduction of Virus Titre in N.glutinosa

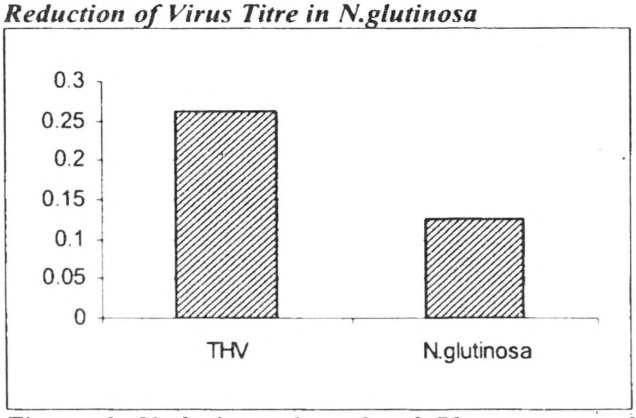


Figure 6- N.glutinosa inoculated Plant compared with the Threshold Value:

After nine weeks of the inoculation N.glutinosa plants were negative although they were positive after five weeks of inoculation. High temperature in the plant house could have reduced the titer of the virus in the inoculated plant (CIP training manual, section 2.2.3:12). Therefore the use of *N.glutinosa* as a source of the virus was unsuccessful in the latter part of the study. Positive and negative controls of Potato were used instead.

CONCLUSIONS

For a detection protocol of PVY using Indirect ELISA, Samples should be extracted by using 0.5 M Potassium phosphate + 0.1 M EDTA+1 percent Sodium sulphite buffer (pH 7.5). The antiserum obtained from the first bleed that was absorbed with partially purified healthy sap given the best. The antiserum should be diluted 1:400 to 1:600 in PBS-TPO. The incubation period should be two hours after adding p-nitro phenyl phosphate substrate.

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ANNEX

Phosphate Buffer Saline-Tween (PBS-T)

8.0 g of Sodium chloride (NaCl), 0.2 g Monobasic potassium phosphate (KH_2PO_4), 1.15 g Dibasic sodium phosphate, 0.2 g Potassium chloride (KCl), 0.2 g Sodium azide (NaN_3), 0.5 ml of Tween-20 were dissolved in 900 ml of distilled water, pH was adjusted to 7.4 and volumerized up to 1000 ml.

PBST+2 percent PVP

20 g of Polyvinylpyrrolidone dissolved in 1000 ml of PBST.

PBS-TPO

2 g of Chicken albumin dissolved in 1 l of PBST+2 percent PVP.

P-nitrophenyl Phosphate Substrate Buffer

0.2g Magnesium chloride ($MgCl_2$), 0.2 g Sodium azide (NaN_3),97.0 ml Diethanol amine was dissolved in 800 ml of distilled water, pH was adjusted to 9.8 and volumerized up to 1000 ml.