Production of Polyclonal Antiserum for Potato Virus X and Development of Detection Method by ELISA.

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ABSTRACT

Potato Virus X (PVX), Potato Virus Y (PVY) and Potato Leaf Roll Virus (PLRV) are the most common viruses found in potato cultivations in Sri Lanka. This research was conducted to develop a low cost detecting method for PVX virus by Enzyme Linked Immunosorbent Assay (ELISA). The plant sap extracted from a selected diseased sample was inoculated to *Nicotiana benthamiana* plants through mechanical inoculation using "Carborandom 400" powder as an abrasive. Plants with systemic symptoms were harvested three weeks after inoculation. The virus was purified according to CIP Training Manual 5.0 Virus Purification, with minor modifications. Purified virus was injected to a Rabbit at one week intervals in six concentrations (0.75mg, 1mg, 1.50mg, 1.63mg, 1.63mg, and 0.84mg). First injection was given intervainally and other injections were given intramuscularly with equal volumes of incomplete Fruend's adjutants. First bleed was collected one week after the third injection to test the development of antibodies against the PVX and other two were collected one and two weeks after the 6th injection. Serum was separated and preserved by adding equal volume of glycerol and 0.025% Sodium azid (NaN_2). Indirect ELISA was done using locally developed antiserum, through optimizing extraction buffer, tissue dilution, antiserum dilution and conjugate dilution. Best extraction buffer was PBST +2%PVP (that maintained plant tissue to buffer ratio as 1:15). Best antiserum dilution was 1:3000 in PBS-TPO buffer and the conjugate dilution was 1:2000 in PBS-TPO buffer. This study revealed that developed potato Virus X antiserum is suitable for routine virus indexing.

KEY WORDS: Potato Virus X (PVX), Polyclonal Antiserum, Enzyme Linked Immunosorbent Assay (ELISA)

1. INTRODUCTION

Potato is the fourth largest food crop in the world. Potatoes are grown in almost all the countries. It is also an important crop in Sri Lanka with reference to consumer preference and net profit. However, potato needs a heavy investment during cropping period in which seed materials alone accounts for about 50%-60% of the total cost of production. The potato farmers in Sri Lanka are compelled to use low quality seed tubers available in the market at cheaper rate or produced by them over several generations using the same seed stock. This results continuous accumulation and spread of various tuber borne diseases as well as consequent yield reduction. Among those diseases, viruses are the most common diseases in Sri Lanka. At present, several viral diseases have been reported in Sri Lanka namely Potato Virus X (PVX), Potato Virus Y (PVY), Potato Leaf Roll Virus (PLRV), Potato Virus A (PVA), and Potato Virus S (PVS). Out of them PVX, PVY and PLRV are the viruses which cause severe damages to potato crop in Sri Lanka.

PVX infection causes latent mosaic or mottle. There are several strains of PVX, which vary to a certain extent in symptom expression and pathogenicity. The virus can be transmitted by sap or by infected propagules. Presence of virus is detected by serological tests and by indicator plants such as *Gomphrena globosa* L. or *Datura strmonium* L. Electron microscopic pictures have indicated that these virus particles are long, flexuous rods, possibly 500-600mμ in length (Smith,1957). Recent research indicates that this virus is transmitted by cultivators and spray equipments too (Manzer and Merriam 1961).

Prevention of the spread of viral diseases is obtained with the use of virus free planting material. Selection of mother stocks based on the symptoms alone is not adequate as these viruses do not show specific symptoms as primary infection and the development of the symptoms depend on the various factors such as variety and the environmental conditions.

Sample testing using serological methods such as enzyme linked immunosorbent assay (ELISA) requires high foreign exchange because most of the commercially available test kits are imported ones and the cost of those kits are very high. Usage of these kits to check the health status of mother plants is not cost effective. Therefore, production of virus detection kits locally is more helpful for the establishment of virus free foundation stock of potato in Sri Lanka.

Therefore, this research was conducted to produce polyclonal antisera for PVX and to develop effective detection method by ELISA

2. MATERIALS AND METHOD

The research investigation was carried out at the Plant Virus Indexing Centre, Department of Agriculture, Gabadawaththa, Homagama. Sri Lanka, from November 2004 to June 2005 and antibody was raised done at the Medical Research Institute (MRI), Borella. Sri Lanka.

2.1. Diseased sample collection and identification of available viruses.

Diseased potato samples were collected from potato growing areas such as Bandarawela and Nuwara Eliya. Those samples were indexed by ELISA with commercially available test kits (DSMZ) to identify the type of viruses available.

2.2. Inoculation and multiplication of Potato Virus X

Two point two grams (2.2g) from 1-9 potato sample (Table-01) was homogenized with 22 ml of extraction buffer that consisted of 0.1 M Phosphate buffer and 0.01M EDTA at pH 7.1. 'Carborandom-400' mesh powder was dusted on to top most four mature leaves of 21 selected *Nicotiana benthemiana* plants. Inoculation was done on the leaves by slightly rubbing with fingers. After the inoculation, leaves were washed with tap water using a hand sprayer and covered with damp newspapers. After incubating for overnight, the moist covers were removed and plants were maintained in an insect proof plant house.

2.3. Purification of Potato Virus X

Nicotiana Benthemiana leaves with systemic symptoms were homogenized with two volumes (w/v) of a buffer solution which contained 0.1M phosphate buffer pH 8 with 0.2% of 2-mercaptoethanol and 10% ethanol. The homogenate was filtered through three layers of gauze and the filtrate was centrifuged at 7800g for 20 minutes. After words the supernatant was processed by adding Triton X-100 to a final concentration of 1 % (v/v) and stirred for one hour at 4 ^{0}C. The solution was centrifuged at 5500g for 20 minutes. NaCl of 0.2 M and Polyethylene glycol (M.Wt 6000) were added to the supernatant to a final concentration of 4 % (w/v). The solution was stirred at 4 ^{0}C for one hour and incubated for another hour at room temperature. Then, the solution was centrifuged at 7800g for 30 minutes and the pellet was resuspended in a solution containing 0.05M phosphate buffer pH 8 with 1 % Triton X-100. The solution was clarified by centrifugation at 3000 rpm for 10 minutes and the supernatant was centrifuged on a 30 % sucrose cushion in 0.05M phosphate buffer pH 8 at 72,500 g for 150 minutes. The pellet was resuspended in a 0.05M phosphate buffer pH 7.2 and stirred for overnight. Then the solution was centrifuged at 2000g for 5 minutes. The supernatant was subjected to one cycle of differential centrifugation and purified further by sucrose gradient (10 to 40%, w/v) in 0.01M phosphate buffer pH 7.2 with 0.01M EDTA in a fixed angle rotor at 96,500g. Both the pellet and cloudy layer of virus band were collected and diluted in the same volume of 0.1 M phosphate buffer pH 7.2. The solution was centrifuged at 102000g for 60 minutes. The pellet was then resuspended in 0.01M phosphate buffer pH 7.2. The absorbance was measured at 260nm and the virus concentration of the purified sample was calculated. This sample was used for the immunization of Rabbit to produce antibodies against PVX.

2.4. Immunization of Rabbit

From the purified virus sample, a sub sample of 0.75mg was diluted up to 0.5ml with 0.01M phosphate buffer pH 7.2. It was mixed with an equal volume of 0.85% NaCl solution and injected to Rabbit intervainally through marginal ear vain. One milligram (1mg) and One point five (1.5mg) from 0.5ml virus suspensions were emulsified with an equal volume of incomplete Fruend's adjuvants and the mixture was injected intramuscularly into the back leg of the Rabbit, using 1ml of the mixture each time as second and third injections. Then, 1.63mg in 1ml virus suspensions were emulsified with an equal volume of Fruend's incomplete adjuvants and the mixture was injected intramuscularly into the back leg of the rabbit using about 2ml of the mixture each time as the fourth and fifth injection and 0.84mg in 0.55ml virus suspension was injected as previously done in the fifth injection, at the final boost. Total of six injections were done to immunize the rabbit with 1-week intervals. The rabbit was bled 3 times at 1 week after the 3rd injection, one and two weeks after 6th injections.

2.5. Antiserum preparation.

2.5.1. Antiserum separation and storage.

Blood was incubated at room temperature for one hour and then it was again incubated at 4 ^{0}C overnight. Then the surrounded serum was transferred to a centrifuge tube and centrifuged at 5000 rpm for 10 minutes. An equal volume of glycerol was added for further processing of the supernatant. Antiserum was stored in refrigerator at –20 ^{0}C after adding of sodium azid (0.025% w/v).

2.5.2. Removal of cross reacting antibodies by absorption.

Healthy *Nicotiana benthemiana* leaves were crushed using sample extractor and the crude sap was collected. An equal volume of antiserum was mixed and incubated for three hour at room temperature. The mixture was then centrifuged at 10000 rpm for 10 minutes. The supernatant was taken as purified polyclonal antiserum for ELISA test for the detection of PVX.

2.6. Optimization of conditions to detect PVX by ELISA.

2.6.1.Optimization of sample extraction buffer

Two healthy samples and two diseased samples were extracted using five common extraction buffers. ELISA test was carried out keeping other conditions constant for each of the extraction buffer. Results were analyzed and the buffer that gave the best results was selected.

2.6.2. Optimization of plant tissue dilution ratio.

Two healthy samples and four diseased samples were crushed and six dilutions of the plant sap were made as 1:2, 1:5, 1:10, 1:15, 1:20, and 1:25 in extraction buffer (PBST + 2% PVP). ELISA test was carried out keeping other conditions constant for each tissue dilution. The results were analyzed and the best dilution which showed best results was selected.

2.6.3. Optimization of best antiserum dilution.

Crude sap was extracted from three diseased and three healthy samples and each sample was diluted in extraction buffer (PBST+2%PVP) with 1:15 dilution. ELISA test was carried out with six antiserum dilutions (1:1000, 1:1500, 1:2000, 1:2500, 1:3000, and

1:3500). The results were analyzed and the best antiserum dilution was selected.

2.6.4. Optimization of best conjugate dilution.

Crude sap of three diseased samples and three healthy samples were extracted and the sap was diluted in extraction buffer (PBST+2%PVP) with 1:15 ratio. The ELISA test was carried out with 1:3000 antiserum dilution and with six conjugate dilutions (1:1000, 1:2000, 1:2500, 1:3000, 1:3500 and 1:4000). After analyzing results, the best conjugate dilution was selected.

3. RESULTS AND DISCUSSION.

3.1. Identification of viruses found in the collected potato samples.

The viruses found in collected potato samples were identified using commercially available test kits. The potato samples were checked for six viruses and only in three viruses were found. PVX, PVY and PVA were detected (Table 01). However the presence of PVA in Sri Lanka was not reported previously. High concentration of PVX is available in samples 1-1 and 1-9. Thus the sample 1-1 contains higher concentration of potato virus Y and it has been infected with PVA too. Therefore, sample 1-9 was used to extract the potato virus x for inoculation of *Nicotiana benthemiana* plants.

Table 01. Identified viruses in collected potato samples.

Sample Number	Available Viruses					
	PVX	PVY	PLRV	PVA	PVS	PVM
1-1	+++	+++	-	+	-	-
1-2	+	++	-	-	-	-
1-3	+	-	-	-	-	-
1-4	+	-	-	+	-	-
1-5	+	+++	-	+	-	-
1-6	-	++	-	-	-	-
1-7	+	+++	-	-	-	-
1-8	-	++	-	-	-	-
1-9	+++	+	-	-	-	-
1-10	-	-	-	-	-	-
1-11	+	++	-	-	-	-
2-1	+	+++	-	-	-	-
2-2	+	+++	-	-	-	-
2-3	-	+++	-	-	-	-
2-4	-	-	-	-	-	-
2-5	+	++	-	-	-	-

+ Positive ness of test results, - Negative test results

3.2. Testing of inoculated Nicotiana benthamiana plants for PVX by ELISA.

An ELISA test was done to check the availability of PVX in the inoculated *Nicotiana benthemiana* plants. A test reading of the ELISA test at 405 nm is shown in the Figure 01.

As seen in the Figure 01 healthy *Nicotiana benthemiana* and healthy potato samples (T1 and T2) have low absorbance values. The positive controls of the test kit (T3) and diseased potato sample (T4) has also shown low absorbance values. However the inoculated *Nicotiana benthemiana* sample (T5) gave the highest absorbance value which indicate that inoculation has been successful.

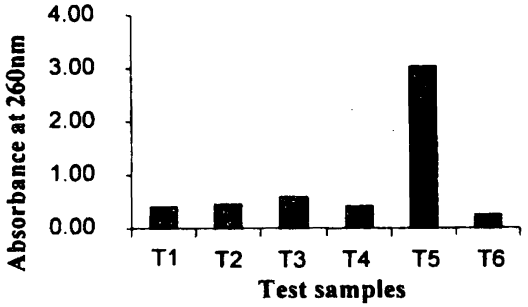

Fig. 01. ELISA test readings of inoculated plants.

T1–Healthy *Nicotiana benthemiana*, T2–Healthy potato sample, T3–Positive kit control, T4–Potato sample (1-9), T5–Inoculated Nicotiana benthemiana ,T6–Extraction buffer

3.3. Purification of PVX from inoculated Nicotiana benthemiana plants.

Three purifications were done to obtain the adequate amount of purified virus. Details are shown in table 02 and the absorbance values were measured using spectrophotometer. Concentration of the virus sample was estimated using specific absorbance (1mg/ml, 1cm light path at 260nm) as 2.9, the value which was used for pure PVX (Leiser and Richter, 1978). However, only the partial purification of virus was done in this study. The estimated concentration of the virus sample was used for immunizing the Rabbit.

3.4. Immunization of Rabbit

An ELISA test was done using the antiserum prepared from the first bleed to check the development of antibodies against the PVX. The ELISA readings are shown in Figure 02.

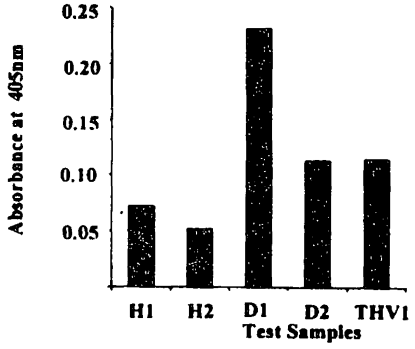

Fig. 02. Testing of antibody development for PVX in Rabbit blood

H1-Absorbance value of healthy sample 1, H2-Absorbance value of healthy sample 2, D1-Absorbance value of diseased sample 1, D2-Absorbance value of diseased sample 2, THV2- Threshold Value,

Threshold value 1 (THV1) was calculated with respect to the absorbance value of healthy sample 1 (H1). When compared to the THV1, diseased samples (D1 and D2) showed positive results while healthy samples (H1 and H2) showed negative results. Positive ELISA results show the presence of specific antibodies for PVX in the serum used. Therefore,

theimmunization has been successful as the antibody was developed in the Rabbit blood.

Table-02, Details of the purified virus samples (from *Nicotina benthamiana* plants)

Purification	Weight of leaf sample	Volume of purified virus	Absorbance at 260 nm	Absorbance at 280 nm	Virus concentration	Total virus yield
First purification	110g	1.2ml	0.456	0.454	3.07 mg/ml	3.68 mg
Second purification	100g	1.5ml	0.323	0.325	2.18 mg/ml	3.26 mg
Third purification	37g	0.55ml	0.226	0.231	1.52 mg/ml	0.84 mg

3.5. Antiserum preparation.

3.5.2. Removal of unwanted antibodies (Absorption).

To test the produced antiserum, two ELISA tests were done using the same healthy and diseased samples. The readings are shown in Fig.03.

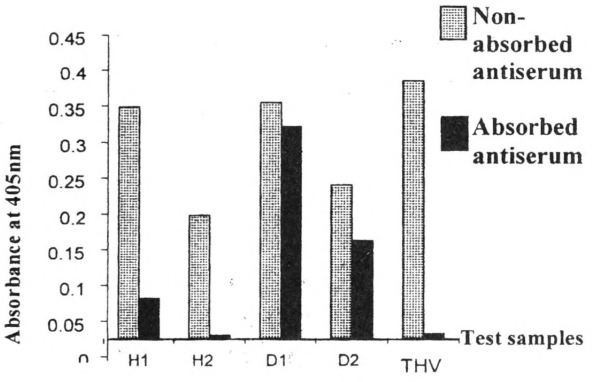

Fig. 03. Comparison of purified antiserum and non Purified antiserum sample.

H1=Absorbance value of healthy sample 1, H2=Absorbance value of healthy sample 2, D1= Absorbance value of diseased sample 1, D2=Absorbance value of diseased sample 2,THV2= Threshold Value

ELISA using the non-purified antiserum gave a high absorbance value for both healthy (H1) and diseased (D1) samples. This is due to the presence of non-specific antibodies in the non-purified serum. In contrast, ELISA using the purified antiserum gave significantly higher absorbance values for both diseased (D1 and D2) samples than for the healthy (H1 and H2) samples. This indicates that the antibody purification step has clearly increased the specificity of the antiserum for PVX by removing the non-specific antibodies by binding with the antigen present in the healthy plant sap.

3.6. Optimization of conditions to detect PVX by ELISA.

The four main conditions (1. Extraction buffer, 2. Plant tissue dilution, 3. Antiserum dilution and 4. Conjugate dilution) involved in indirect ELISA test was optimized by conducting four experiments. Each experiment was carried out changing one condition keeping other conditions constant. Test readings obtained are shown in Fig.04, Fig.05, Fig.06 and Fig.07.

3.6.1. Optimization of sample extraction buffer.

Five extraction buffers were used to prepare the virus samples from the plants and the ELISA results obtained in this experiment are displayed in Fig.04. Buffers A (0.5M Phosphate buffer (pH 8.4) + 5g/l Sodium sulphite) and D (Carbonate buffer) gave negative absorbance values for healthy samples. Buffer C (Agdia Extraction buffer) did not show any difference between the healthy and diseased samples. Buffer B (0.5 M Phosphate buffer pH 7.5 with 0.1M EDTA and 1% Sodium sulphite) and E (PBST + 2%PVP) gave positive values for healthy samples and showed a clear difference between absorbance values of healthy samples and diseased samples. Buffer E was identified as the most suitable one since it had the highest indexing power as estimated by D1/THV.

3.6.2. Optimization of plant tissue dilution

Six plant tissue dilutions in extraction buffer E were used for ELISA to determine the optimum tissue dilution for ELISA tests. Fig.05 shows the results of this experiment. The highest absorbance value of diseased samples was observed in 1:2 tissue dilutions and the absorbance values have decreased with increasing plant tissue dilutions. The highest indexing value for PVX was obtained with 1:15 plant tissue dilution

3.6.3. Optimization of Antiserum dilutions in PBS-TPO

The results obtained with different antibody dilutions are given in Fig.06. There was no significant difference between the absorbance values of diseased samples. The 1:3000 antiserum dilutions gave the highest D1/THV value and were therefore, selected for ELISA for the detection of PVX.

3.2.4. Optimization of conjugate dilution in PBS-TPO

The results of the ELISA experiment using different conjugate dilution were given in Fig.07. Higher absorbance values for diseased sample were observed with higher concentration of conjugate. However, the highest value of D1/THV was obtained in 1:2000 conjugate dilution even with considerably low absorbance values for diseased samples. Therefore the best conjugate dilution was identified as 1:2000 for ELISA test.

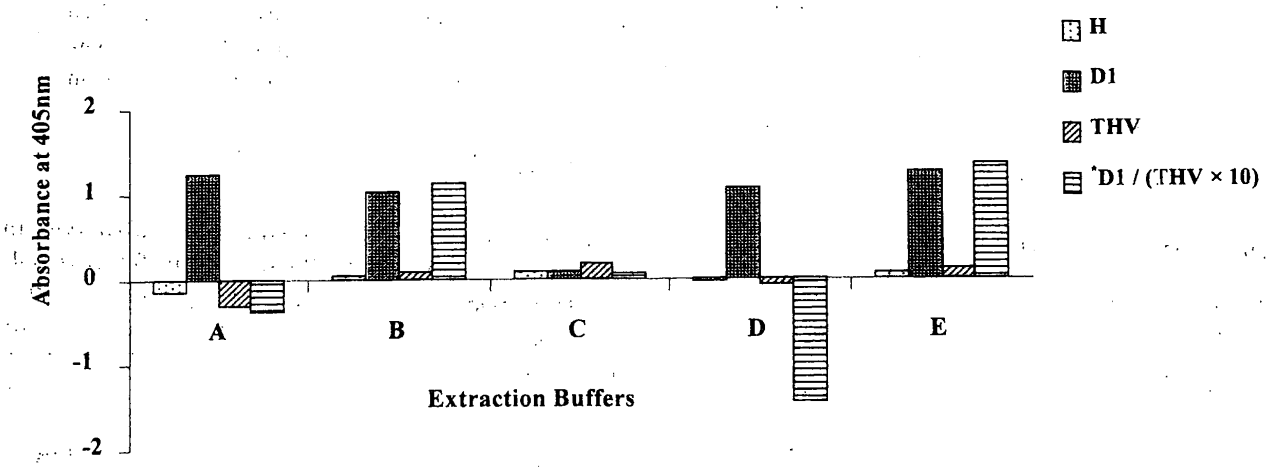

Fig:-04, Optin *** This figure (D1/THV) indicates the power of the test of sample absorbance compared to the THV**

A-0.5M Phosphate buffer(pH 8.4) + 5g/l Sodium sulphite,B-0.5 M Phosphate buffer pH 7.5 with 0.1M EDTA and 1% Sodium sulphite, C-Agdia Extraction buffer, D-Carbonate buffer,E-PBST + 2%PVP,H-Absorbance value of healthy samples,D1-Absorbance value of diseased samples,THV- Threshold Value

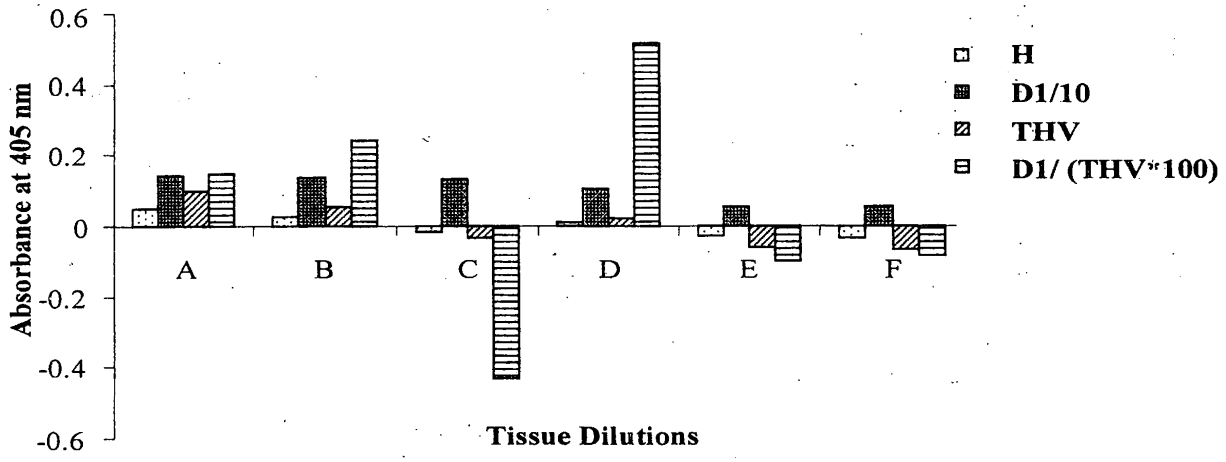

Fig.05 Optimization of plant tissue dilution

A-1:2 Plant tissue dilution in Extraction buffer, B- 1:5 Plant tissue dilution in Extraction buffer, C- 1:10 Plant tissue dilution in Extraction buffer, D- 1:15 Plant tissue dilution in Extraction buffer, E- 1:20 Plant tissue dilution in Extraction buffer, F- 1:25 Plant tissue dilution in Extraction buffer, H-Absorbance value of healthy samples, D1- Absorbance value of diseased samples, THV- Thresh Hold Value.

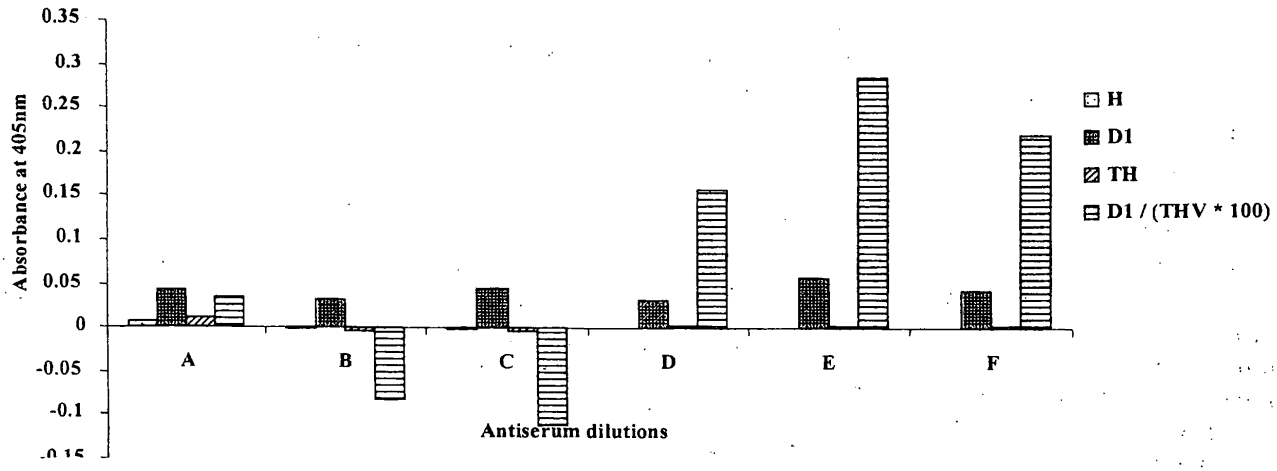

Fig.06 Optimization of Antiserum dilution.

A-1:1000 Antiserum dilution in PBS-TPO, B-1:1500 Antiserum dilution in PBS-TPO, C-1:2000 Antiserum dilution in PBS-TPO, D-1:2500 Antiserum dilution in PBS-TPO, E-1:3000 Antiserum dilution in PBS-TPO, F-1:3500 Antiserum dilution in PBS-TPO,H- Absorbance value of healthy samples,D1-Absorbance value of diseased samples, THV- Threshold Value.

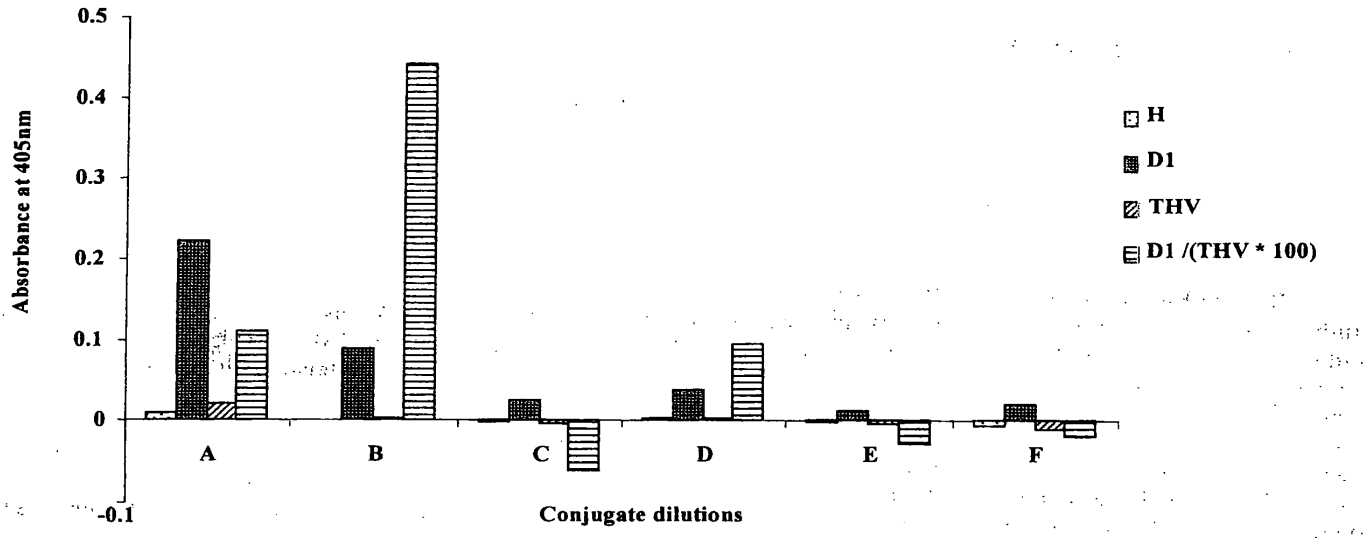

Fig: - 07, Optimization of conjugate dilution

A-1:1000 Conjugate dilution in PBS-TPO,B-1:2000 Conjugate dilution in PBS-TPO, C-1:2500 Conjugate dilution in PBS-TPO, D-1:3000 Conjugate dilution in PBS-TPO, E-1:3500 Conjugate dilution in PBS-TPO, F-1:4000 Conjugate dilution in PBS-TPO, H-Absorbance value of healthy samples, D1-Absorbance value of diseased samples, THV- Threshold Value.

4. CONCLUSIONS.

In preparation of the antibody, the purification step of the antiserum against the healthy *Nicotiana benthemiana* sap increased the specificity of the antiserum to PVX. The produced PVX specific antiserum could be used to detect the PVX infected samples by using ELISA. The best extraction buffer for sample preparation was found to be PBST + 2%PVP. The best tissue dilution, antiserum dilution and conjugate dilution for ELISA were 1:15, 1:3000 and 1:2000 respectively.

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