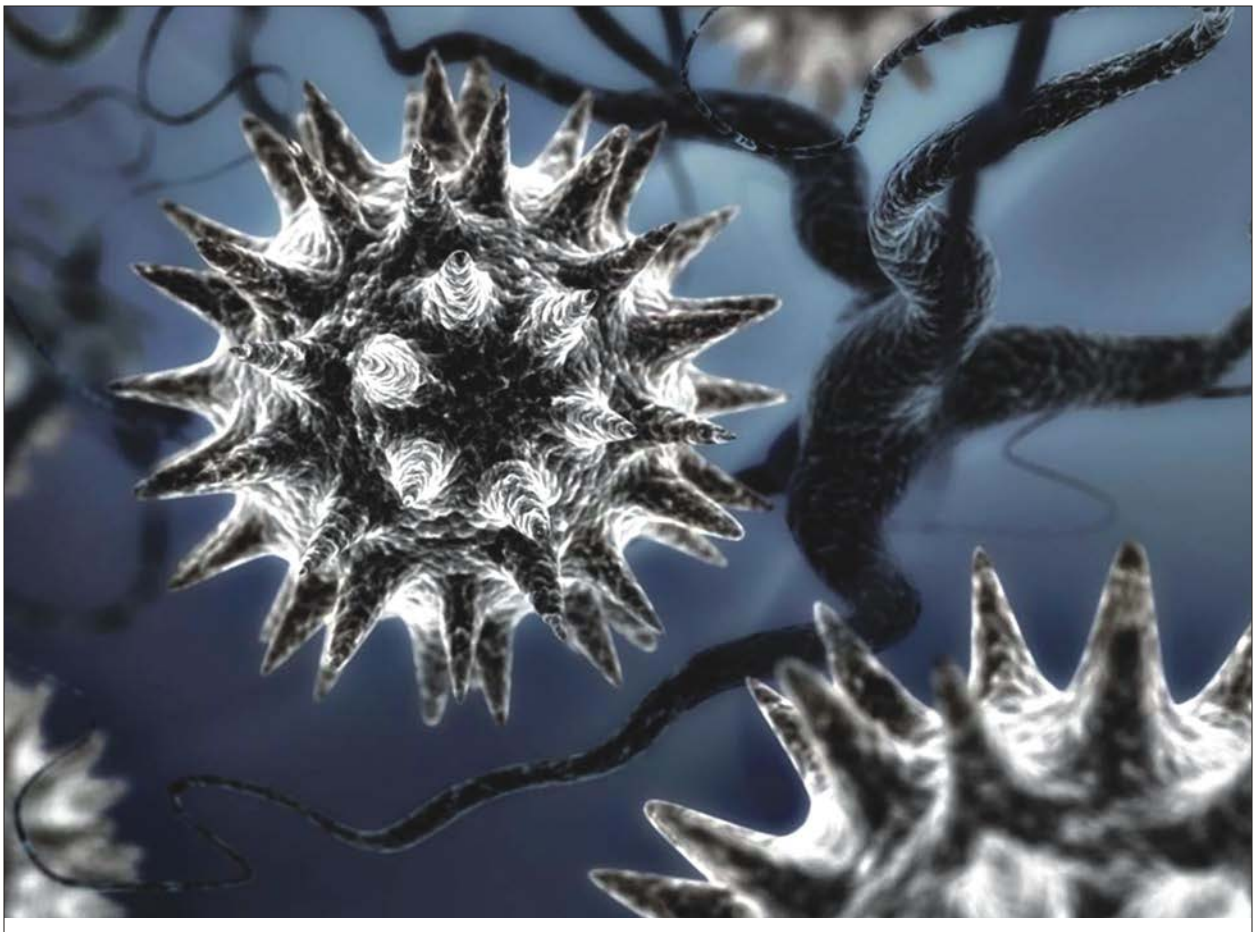

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Production of Polyclonal Antisera to a Recombinant Coat Protein of *Potato virus Y* Expressed in *Escherichia coli* and its Application for Immunodiagnosis

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ABSTRACT

A polyclonal antiserum to a recombinant Coat Protein (CP) of *Potato virus Y* (PVY) was developed and its effectiveness was measured with double antibody sandwich immunosorbent assay (DAS-ELISA), indirect ELISA (I-ELISA), Indirect Plate Trapped Antigen (IPTA) ELISA, Western Blotting (WB) and dot blotting immuno binding assay (DBIA). The CP gene of PVY was amplified with the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using primers, designed from a recombinant CP sequence for PVY^{O} (common strain) and PVY^{N} (the necrotic strain), to amplify the full CP from a mixture of different PVY isolates (PVY^{O}, PVY^{N}, PVY^{NTN} and $PVY^{N:O}$). The full 800 bp-CP amplicon gene was cloned and expressed into pBAD-C terminal 6×His Tag TOPO expression vector in *Escherichia coli* BL21. The CP fraction from bacterial lysates was purified, under native and denatured conditions, by nickel-nitrilotriacetic acid (Ni-NTA) batch chromatography; yielding 0.6 mg mL^{-1}. Antigenicity of the purified CP fraction was measured with Western Blotting (WB) analysis. For immunization, the CP fusion protein was injected into rabbits. The recombinant PVY-CP antiserum reacted with PVY-infected potatoes using IPTA-ELISA and DBIA and with a wide spectrum of local and foreign strains of PVY including PVY^{O}, PVY^{N} and PVY^{NTN} in DAS-ELISA and DBIA. The data indicated that the produced recombinant antiserum was efficient and accurate in determination of negative and positive results in ELISA tests. Therefore, this antiserum is suitable for certification programs of potatoes due to its low cost, high specificity, feasibility and its endless supply from recombinant bacterial clones carrying the CP genes for this virus.

Key words: Potato, *Potato virus Y*, RT-PCR, recombinant coat protein antisera, immunodiagnosis

INTRODUCTION

Potato virus Y (PVY, genus Potyvirus, family Potyviridae) is one of the most economically important viruses of the potato crop worldwide (Singh *et al*., 2008). The virus possesses a single-stranded, positive sense RNA genome of approximately 9.7 kb, with one open reading frame encoding a single polyprotein (Riechmann *et al*., 1992). PVY induces various foliar symptoms in potato plants ranging from mosaic to leaf-drop, streaks and stunting, depending on cultivars and

virus strains and can cause up to 80% yield reduction (Nolte *et al*., 2004; Whitworth *et al*., 2006). Many strains of PVY have been recognized according to the primary hosts and host range reactions including the potato-infecting PVY (De Bokx and Huttinga, 1981). The ordinary strain (PVY^{O}), the tobacco veinal necrosis strain (PVY^{N}) and the potato stipple streak strain (PVY^{C}) were the first ones to be recognized (De Bokx and Huttinga, 1981; Shukla *et al*., 1994). PVY has a high degree of genetic variability and is also subject to recombination (Revers *et al*., 1996; Rolland *et al*., 2009; Hu *et al*., 2011). In the early 1980s, a number of PVY recombinants were documented including the recombinant N: O/wilga group fromNorth America termed $PVY^{N:O}$ and the PVY^{NTN} (Nie *et al*., 2004; Piche *et al*., 2004; Crosslin *et al*., 2005). PVY^{NTN} is a variant of PVY^{N} characterized by its ability to induce Potato Tuber Necrotic Ringspot Disease (PTNRD) and severe chlorotic mosaic in sensitive potato cultivars (Nie and Singh, 2003a, b; Nie *et al*., 2004). In Egypt, several authors reported isolation of PVY^{O} and PVY^{NTN} from potato (Kishtah, 1970; Gamal El-Din *et al*., 1997; Amer *et al*., 2004).

For the production of good quality polyclonal antibodies, an efficient purified virus antigen is required. However, complications can hinder the fidelity of the purification process including low virus titres, virus aggregation, conformational changes of epitopes and mixed virus infections. Low virus titre, during extraction is often associated with the natural presence of certain viruses in phloem tissues (e.g., *Citrus tristeza virus* (CTV) infecting citrus, *Banana streak virus* (BSV) *Banana bunchy top virus* (BBTV) infecting banana, *Grapevine leafroll-associated virus* (GLRaV) infecting grapevine and PLRV infecting potato (Lopez *et al*., 1994; Nikolaeva *et al*., 1995; Abdel-Salam *et al*., 2004, 2005). This in turn necessitates the use of large amount of starting-infected materials and therefore increases the chance of having high background related to plant-host antigens in the induced antiserum. Purification of some viruses such as the PVY group, are difficult due to virus aggregation and its association with plant antigens and the presence of inhibitory compounds such as polyphenols, tannins and polysaccharides resulting in sometimes high background in the used antiserum due to contamination of virus antigens with host proteins (Abdel-Salam *et al*. 1989, 1990; Ling *et al*., 2000; Xu *et al*., 2006; Beuve *et al*., 2007; Fajardo *et al*., 2007). Conformational changes of virus epitopes can occur during purification, as observed with *Grapevine virus B* (Boscia *et al*., 1993) and partial degradation of antigens due to proteolysis occurring during storage of sugar beets (Narayanasamy, 1997) can lead to low immunogenicity of the antiserum and subsequently affects serological detection of those viruses. The presence of multiple virus species can lead to co-purification of other viruses and hence production of mixed antibodies therefore, affecting virus diagnosis. For instance, the *Grapevine leafroll-associated virus* (GLRaV) has nine virus species associated with the disease complex which are inseparable by biological means, with the exception of GLRaV-2. This in turns renders differentiation between these species almost impossible serologically with an antiserum induced by the classical means (Fajardo *et al*., 2007). Many plant viruses are labile in nature as in members of the *Ilarvirus* group. This leads to problems in purification which affects the specificity of the induced antisera (Abdel-Salam *et al*., 2008a, b).

Recombinant DNA technology may help circumvent purification problems encountered with several viruses (Ling *et al*., 2000; Abdel-Salam *et al*., 2004, 2005, 2008a, b; Beuve *et al*., 2007; Fajardo *et al*., 2007). One of the major advantages of this technology is the high fidelity of the expressed virus-coat protein and subsequently high specificity antibody production with no cross-reaction to plant proteins. Also the recombinant-protein technology can guarantee an endless supply of antibodies pending the presence of bacterial clones carrying the plasmid insert of the virus coat protein genes.

In this study, we report the use of recombinant DNA technology to produce the CP of PVY *in vitro*, production of a polyclonal antiserum with wide reactivity to PVY strains from this recombinant CP and suitability of the antiserum for use in I-ELISA, IPTA-ELIA, DAS-ELISA and DBIA.

MATERIALS AND METHODS

Virus isolates: Four major symptoms were observed on potato (*Solanum tuberosum* L.) plants infected with PVY in the field including (1) Mosaic, leaf deformation and leaf defoliation on Spunta and Annabella potato verities, (2) Necrotic leaf spot (NLS) symptoms on Sputa potato variety, (3) Severe chlorotic mosaic and necrosis on Anabella potato variety and (4) NLS symptoms plus veinal necrosis and tuber necrosis on Nicola potato variety. Samples from each infected potato variety were collected and tested with DAS-ELISA using PVY antiserum donated by P.E. Thomas, University of Washington, Prosser, WA, USA. Samples tested positive for PVY using this antiserum and showing any of the described four-group symptoms were frozen at -80°C for future use. Compound samples, of leaves with each symptom type were bulked together to form a single sample, were also prepared and frozen or used immediately for nucleic acid extraction and molecular studies.

Coat protein amplification and cloning: Samples of potato cv. Spunta showing severe chlorotic mosaic and leaf necrosis which tested positive for PVY in DAS-ELISA using PVY antiserum were donated by P.E. Thomas and used to amplify the PVY CP by RT-PCR. Total RNA was purified from either frozen or fresh samples of this PVY positive material using the RNeasy® Plant Mini Kit obtained from QIAGEN according to manufacturer's instructions.

To amplify the coat protein of PVY, primers were designed from a sequence of the coat protein gene for PVY N:O-Mb112 (GenBank Accession No. AY745491; Nie *et al.* (2004). The virus sense primer PVY-N:O-f (5'CAAATGACACAATCGATGCAGGAGG3'), corresponds to nt positions 8572 to 8596 of the complete PVY sequence while the complementary sense primer PVY-N:O-r (5'CGACCATGGCATGTTCTTAACTCC3') with NcoI restriction site, underlined, corresponds to nt 9357-9371. The reverse primer was designed to remove the native stop codon in the coat protein genes and preserve the reading frame through the C-terminal tag to include the V5 epitope and the polyhistidine region in the pBAD-TOPO vector.

Complementary DNA (cDNA) was reverse-transcribed from 2 μL of total RNA (0.17 mg mL^{-1}) in a total reaction volume of 20 μL (Cat No. M510A Promega, Madison, WI, USA). The reaction mixture was incubated at 42°C for 1 h then frozen. For PCR analysis, 2 μL cDNA was used in a total reaction volume of 25 μL reaction mixture containing 0.5 μL of each primer (10 μm), 0.5 μL of 10 mM dNTP mix, 5 μL of 5X GoTaq DNA polymerase reaction buffer (Cat No. M8301, Promega, Madison, WI, USA), 2.5 μL of 25 mM $MgCl_2$, 0.5 μL (1 U) of Taq DNA polymerase (M8301, Promerga) and 13.5 μL of water. Reactions were incubated through 35 cycles of 94, 55°C for 30 sec and 72°C for 45 sec, then given a final extension of 5 min at 72°C. Five microlitter aliquots of PCR amplicons were analyzed on 1% agarose gels using 0.5X TBE with a one kb DNA ladder (Promega, USA). Gels were stained with ethidium bromide and visualized by UV illumination.

RT-PCR amplicons were cloned into pBAD-TOPO® vector (pBAD-TOPO® TA Expression Kit, Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The recombinant plasmids were multiplied using the *E. coli* strain BL21 (DE3) as described by the manufacturer's instructions. Plasmid DNA was prepared from selected white colonies, digested with NcoI and fractionated on agarose gels to check insert identities of plasmids.

Expression and purification of the PVY coat protein: To express the PVY CP genes, an *E. coli* BL21 colony containing the desired recombinant plasmid was used to inoculate liquid Luria-Bertani (LB) medium containing 60 μg mL^{-1} ampicillin and incubated overnight at 37°C with shaking at 250 rpm. One milliliter of overnight culture was inoculated into 50 mL of LB medium containing 60 μg mL^{-1} ampicillin, incubated at 37°C with vigorous shaking to reach an OD_{600} of approximately 0.5. One milliliter of culture was centrifuged at 12,000 rpm for 1 min at room temperature and the pellet was stored at -20°C. Expression of the recombinant protein was induced in the remaining culture by adding L arabinose. To optimize induction of the protein, five different final concentrations (0.00002, 0.0002, 0.002, 0.02 and 0.2%) of L arabinose were tested. One millliter aliquots of the induced cultures were removed at 2, 3 and 4 h after induction, immediately centrifuged at 12,000 rpm for 1 min at room temperature and pellets were stored at -20°C. Protein expression for the different concentrations of inducer tested was evaluated by electrophoresis and gel staining as described by Sambrook *et al.* (1989). Briefly, the pellets were re-suspended in 100 μL of 1X SDS gel loading buffer and heated to 100°C for 3 min, prior to separation through a 12% SDS-PAGE and staining with Coomassie Blue. The remainder of the induced cultures were centrifuged at 4,000 rpm for 30 min at 4°C and pellets stored at -80°C for protein purification. Proteins were purified using the 6XHis Spin Purification Kit (Ni-NTA Fast Start Kit Cat No. 30600, Qiagen) as per manufacture's instructions. The eluted 6XHis fusion proteins were assayed on 12% SDS-PAGE as described above.

Large scale protein production and purification: A single bacterial colony transformed with pBAD-N construct was inoculated into 10 mL LB medium supplemented with 60 μg mL^{-1} ampicillin and grown overnight at 37°C. A large volume (250 mL) of LB, supplemented with ampicillin, was inoculated with the 10 mL overnight cultures and grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was reached. A sample of 0.5 mL was taken and kept preserved at -20°C as a non-induced control. Induction of protein expression was obtained by adding 0.0002% of L-arabinose, as final concentration, with shaking (225 rpm) at 37°C for 4-5 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. A 0.5 mL fraction was taken and preserved at -20°C as an induced control. Both un-induced and induced controls were analyzed by SDS PAGE. Cells were harvested by centrifugation at 4000 rpm for 20 min. Pellets were kept overnight frozen at -20°C.

Purification of the induced recombinant proteins was via both native and denaturing conditions in attempts to obtain antigenic viral external and internal CP epitopes for animal immunization. Purifications were performed using the Ni-NTA Fast Start Kit (Cat No. 30600) by Qiagen as per manufacturers'. Pellets from large scale cultures were thawed for 15 min on ice and re-suspended in lysis buffer (pH 8.0) containing lysozyme and benzonase. Suspensions were incubated on ice for 30 min with occasional swirling of the cell suspension and then centrifuged 14,000 g for 30 min at 4°C. Cell lysate supernatants, containing the soluble recombinant protein fractions, were transferred to new tubes. The remainder of the cell lysate was applied to a Fast Start column. The column was washed twice with four ml of wash buffer (pH 8.0) for native purification or pH 6.3 for denaturing purification. Bound 6XHis-tagged proteins were eluted with 3-1 mL aliquots of Elution buffer (pH 8.0, for native purification) or pH 6.3 (for denaturing purification). At each step, a 5 μL sample was collected to monitor the purification process. Each sample was mixed with 2×SDS-PAGE buffers and stored at -20°C for SDS-PAGE. Elutes of recombianant CP, prepared under naturing and denaturing methods, were added altogether and dialyzed overnight at 4°C in 20 mM

phosphate (Na_2HPO_4-NaH_2PO_4) buffer, pH 7.4. Protein concentration of the recombinant PVY-CP was estimated by spectrophotometry using an extinction coefficient = 1 (A_{280} = 1 for 1 mg mL^{-1}).

In some experiments, yield of the recombinant proteins was very low. To overcome this problem, elutes of recombinant protein were combined and dialyzed overnight at 4°C in 1 mM Tris buffer, pH 8.0. The dialyzed proteins were then concentrated by the electro elution apparatus (Little Blue Tank, ISCO Scientific) using 20 mM phosphate buffer, pH 7.4 and 4 mA $cell^{-1}$ for 30-60 min.

Western blot analysis: Proteins separated by SDS-PAGE were electroblotted onto nitro cellulose membrane (0.45 μm, Biobind-NC, Whatman, England) in a semidry system (Biometra, Germany) using 2.5 mA cm^{-2} using a transfer buffer (25 mM Tris-base, 150 mM glycine and 10% methanol, pH 8.3). After blotting, the membrane was stained non-specifically with Ponceau S (Sigma-Aldrich, St Louis, Mo, USA). The membrane was then incubated for 1 h at 37°C in 5% non-fat dry milk and 1% bovine serum albumin, prepared in PBST, pH 8.0. For the detection of the recombinant/CP, the membrane was incubated overnight at 4°C with PVY antiserum (P.E. Thomas), diluted 1/100 in PBS, or 1/250 of the recombinant PVY-IgG (this study) diluted in ELISA coating buffer (15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). The membrane was washed four times in PBST and incubated for 3 h at 37°C in alkaline phosphatase conjugated goat anti rabbit IgG (Sigma-Aldrich, A-3687, St Louis, Mo, USA) diluted 10^{-4} in ELISA conjugate buffer (PBST, pH 7.4, containing 0.2% BSA and 2% polyvinyl pyrrolidone, PVP). The membrane was washed six times in PBST to remove non-bound secondary antibodies. The bands of interest were visualized by the Fast Red TR/Naphthol/naphthol AS-MX-phosphate complex (Sigma-Aldrich, A-3687, St Louis, Mo, USA).

Production and purification of antisera: Immunization of a rabbit involved injection with an equal mix of fusion CP prepared under native and denaturing purification conditions and suspended in 20 mM phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.1% Triton X100. The animal received five weekly consecutive intramuscular injections. Each injection was composed of 600 μg fusion CP mixed (1:1, v/v) with Freund's complete adjuvant in the first-week injection. Subsequent injections used incomplete adjuvant instead. One week after the last injections, the rabbit was bled. The serum fraction of the blood was separated through centrifugation at 1300 rpm for 15 min at 4°C then stored at -20°C. The IgG fraction was precipitated from the crude antisera by caprylic acid and ammonium sulphate according to the method of Mckinney and Parkinson (1987). The concentration of IgG was measured at 280 nm (OD of 1.4 = 1 mg mL^{-1}) with 0.5×PBS The measured IgG was frozen at -20°C. Alkaline phosphatase (AP)-IgG conjugate was prepared following the one-step gluteraldhyde method as described by Converse and Martin (1990). The AP-IgG conjugate was preserved in 50% glycerol, 0.02% NaN_3 and stored at 4°C.

Immunoassay tests: Indirect (I)-ELIA was used according to Converse and Martin (1990) for measuring titre of the PVY-recombinant antiserum. Samples of Spunta potato cv. plants infected with PVY were diluted 1/10 (w/v) in conjugate buffer. Microtitre plates were blocked for 30 min at 37°C with 5% non-fat dry milk powder prepared in PBST containing 2% PVP and ten three fold-ascending dilutions of the tested antitserum (up to 7×10^{-3}) in PBST were added to the plate and incubated overnight at 4°C. Bound antigen was detected with goat anti-rabbit (GAR)-IgG-AP conjugate (Sigma A-8025) diluted at 10^{-4} in conjugate buffer. The plates were washed with PBST

between each step and final detection was via addition of 1.0 mg mL^{-1} para nitrophenyl phosphate (PNPP) substrate, dissolved in diethanolamine buffer (pH 9.8) and measurement of colour development.

Indirect Plate-Trapped Antigen (IPTA)-ELISA was used to detect potato infected plants using recombinant coat protein antisera according to Folwarczna *et al.* (2008). Microtitre plates were coated with antigens diluted 1/10 in standard carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. The ELISA plates were washed four times with PBST and incubated for 2 h at 37°C with 4 μg mL^{-1} PVY-IgG diluted in conjugate buffer. The plates were washed and incubated with GAR-AP conjugate as described previously. Plates were given 6 times washing prior to substrate addition.

Double Antibody Sandwich (DAS)-ELISA was used to test the presence of PVY isolates in different potatoes. The test was also used to measure the specificity of the recombinant CP antiserum against local and foreign isolates of PVY. To test specificity and sensitivity of the antiserum, the IgG was tested at three different concentrations (4, 2 and 1 μg mL^{-1} against different dilutions of recombinant PVY-IgG labeled AP (1/250, 1/500, 1/1000 up to 1/10,000). This was done essentially as described by Clark and Adams (1977) with the following minor modifications. After coating the plates with the different IgG concentrations, the plates were blocked for 1 h at 37°C using 5% non-fat dry milk (w/v) and 1% (w/v) BSA.

Dot blotting immunosorbent assay, DBIA, was used to confirm detection of the expressed PVY-CP antigens and to detect PVY isolates in infected potato, tobacco and tomato tissues. To do DBIA, nitrocellulose membranes (NCM) (0.45 μm-Bio-Bind-NC Whatman) were soaked in distilled water for 5 min and then air dried. Antigens were ground in coating buffer pH 9.6 (1:10, w/v) and then blocked for 1 h at 37°C in 5% non-fat dry milk prepared in PBST. NCM were incubated for 2 h at 37°C with PVY-IgG diluted 1/250 in PBST, washed and then incubated for 2 h at 37°C with GAR-AP conjugate diluted at 10^{-4} in conjugate buffer. The membrane was washed 6 times in PBST with each wash being 5 min in duration between each antiserum step. The blots were then incubated in substrate solutions consisting of NBT/BCIP complex or Naphthol/Fast Red complex for violet or red color development, respectively as described by Lizarrage and Fernandez-Northcote (1989) and Abdel-Salam *et al.* (1997).

RESULTS

Virus symptoms: Symptoms caused by PVY infection can vary depending upon the strain and grown potato variety. Four groups of symptoms were observed on tested potato in the present study. Group 1 symptoms included leaf mosaic, leaf malformation and defoliation on Spunta and Annabella potato varieties and mimicked PVY^{O}-induced symptoms on potato (Fig. 1). Group 2 symptoms were observed on infected Spunta potato variety. These included the formation of leaf-necrotic spots with no detected symptoms on formed tubers (Fig. 2a). These symptoms are similar to symptoms caused by the PVY^{N} on potato. Group 3 symptoms appeared on Annabella potato variety and involved the formation of severe chlorotic mosaic and leaf necrosis (Fig. 2b). Such symptoms may represent mixed infection of potato with and PVY^{N} and PVY^{O} or infection with the recombinant $PVY^{N:O}$ strain. Group 4 symptoms were observed on Nicola potato variety and included the formation of necrotic spots and veinal necrosis coupled with the development of necrotic ringspots on tubers after 30-45 days of storage (Fig. 3). These latter symptoms are similar to those developed by PVY^{NTN}.

Fig. 1(a-c): (a) Group 1 symptoms of the PVY^{O} mosaic strain on Spunta potato variety. Infection of potato leads to mosaic development, (b) Leaf malformation and (c) Leaf defoliation giving the palm-tree-like shapes

Fig. 2(a-b): Symptoms of PVY infection (a) Group 2 symptoms: Necrotic ring spots on Spunta potato variety infected and (b) Group 3 symptoms: Severe chlorotic mosaic and necrotic spots on leaves of Annabella potato variety

Fig. 3(a-b): (a) Group 4 symptoms of necrotic ring spots and veinal necrosis and (b) Necrotic tuber ring spots on Nicola potato variety infected with PVY isolate(s)

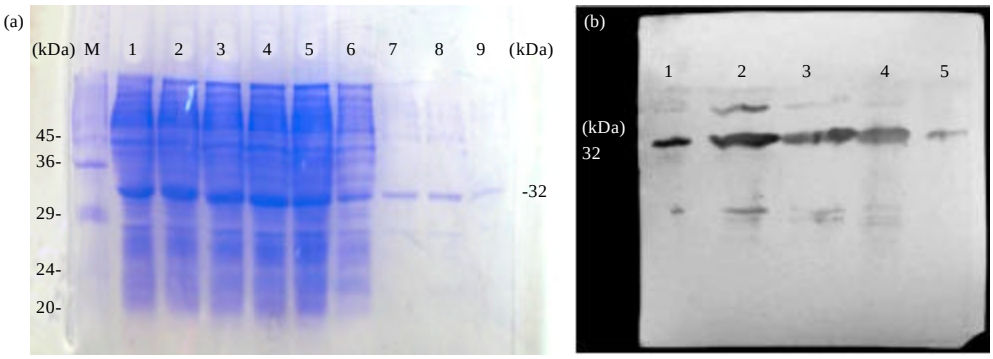

Fig. 4(a-b): Evaluation of expressed recombinant CP by (a) SDS-PAGE gel stained with Coomassie staining solution where Lane M: SIGMA low molecular weight protein marker No. M-3913, Lane 1-5: are total protein from cultures induced with L arabinose at final concentrations of 0.00002, 0.0002, 0.002, 0.02 and 0.2%, respectively, Lane 6: An un-induced control and Lane 7-9: Elutions 1, 2 and 3, respectively, of recombinant PVY-CP after Ni-NTA chromatography and (b) Western blot analysis of a parallel run of SDS-PAGE expressed recombinant CP where Lane 1: Un-induced control, Lane 2: 0.0002% L arabinose induced control and Lane 3-5: Eluted fractions 1, 2 and 3, respectively, of purified PVY CP after Ni-NTA chromatography

Generation of recombinant PVY-CP antiserum: Primers for the amplification of the PVY CP were designed from a recombinant sequence of the coat protein gene for PVY N:O-Mb112 recombinant isolate (GenBank Accession No. AY745491; Nie *et al.* (2004). The reverse primer had one NcoI restriction site to allow direct cloning into the pBAD-TOPO vector. The RT-PCR amplified the full ca 800 bp CP for the total PVY-RNA extracted from a sample containing a complex of leaf mosaic and necrosis symptoms. The PVY-CP plasmid construct was used to transform *E. coli* BL21.

The CP gene for PVY in the recombinant plasmid was expressed in *E. coli* with L arabinose and purified under native and denaturing conditions. The non-induced culture produced a lower amount of protein than induced ones as expected. The total yield of CP fusion protein fractions, from both methods, was 0.6 mg mL^{-1}. An SDS-PAGE analysis of these fractions shows optimum increasing of protein yield with 0.0002% concentration of the inducer (Fig. 4a). The purified fusion

CP measured 32 kDa in SDS-PAGE which is consistent with that reported for PVY-CP by Amer *et al.* (2004). Other authors reported molecular weight values for PVY-CP ranging from 29-34 kDa according to the chosen purification procedures (Huttinga and Mosch, 1974; Van der Vlugt *et al.*, 1988; Watts and Singh, 1994; Folwarczna *et al.*, 2008).

The antigenicity of the induced PVY-CP was validated with WB analysis and DBIA analysis. In WB analysis, the polyclonal antibodies reacted with purified CP band of 32 kDa which is the expected molecular weight of PVY-CP (Fig. 4b). Furthermore, two additional smaller minor bands were detected in both non-purified and purified fractions of the expressed CP.

Evaluation of the recombinant PVY-CP antiserum for virus detection: The recombinant PVY-CP antiserum was evaluated by measuring its titre and efficacy in detecting PVY using: I-ELISA, IPTA-ELISA, DAS-ELISA and DBIA tests. The measured titers, with I-ELISA, expressed as reciprocal of the dilution endpoints was 6000. This method was previously shown to be useful in detection of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* with their recombinant antisera (Lee and Chang, 2008).

IPTA-ELISA was successful in detection of PVY in leaves and tuber sprouts of several potatoes varieties (Fig. 5a). IPTA-ELISA was used by several investigators for evaluating recombinant antisera of *Potato mop top virus* in potato (Cerovska *et al.*, 2003) and PVY in potato (Folwarczna *et al.*, 2008). DAS-ELISA was used to test isolates of PVY in the field using its recombinant antiserum because of its high selectivity. Several concentrations of IgG and IgG-AP conjugate were tested for DAS-ELISA optimization. Dilutions 1/250 and 1/500 were the optimal dilutions for the IgG and IgG-AP conjugate, respectively. Results shown in Fig. 5b indicate that

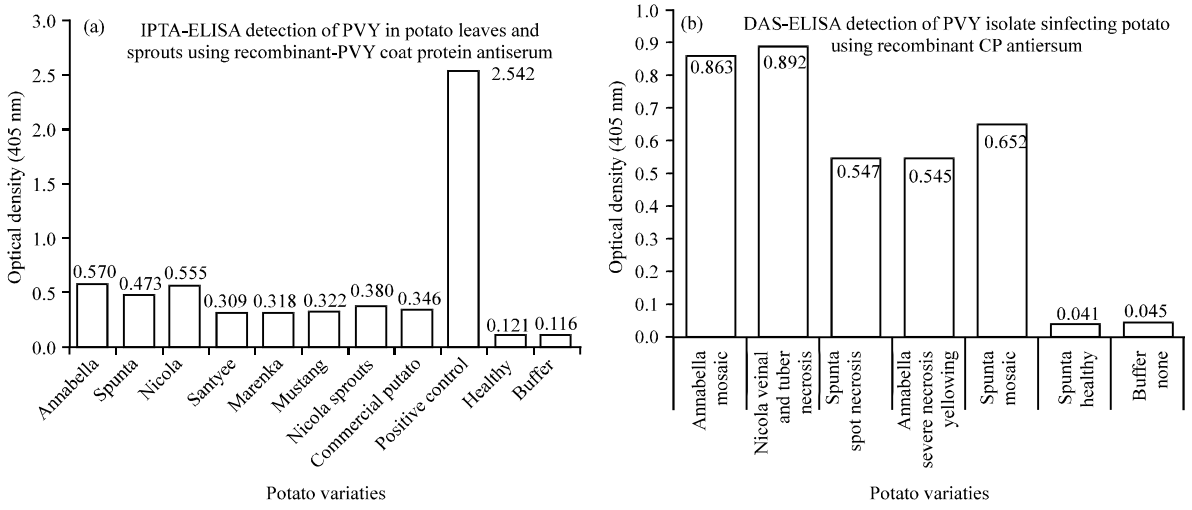

Fig. 5(a-b): Serological evaluation of expressed recombinant CP by (a) IPTA-ELISA and (b) DAS- ELISA. A range of PVY infected potato leaves and sprouts from various varieties were tested by IPTA-ELISA and each reading is an average from ten replicates. For this assay the IgG dilution was 1/250 and the GAR-AP dilution 10^{-4} and a positive result was taken as exceeding 2.5 times the mean of the corresponding healthy control. The positive control was the PVY fusion CP diluted 10^{-1} and is equivalent to 60 μg mL^{-1}. R^2 = 0.0116. For the DAS- ELISA assay, three potato varieties infected with different PVY isolates and showing different symptoms were tested using the PVY recombinant-CP antiserum

different PVY isolates varied in virus accumulation for a given potato variety. For instance, virus concentrations varied in the Annabella potato variety depending on the invading virus isolate. Despite the high specificity of DAS-ELISA, the test was able to detect several PVY isolates because the PVY recombinant-CP antiserum was designed from a recombinant sequence for the CP of several potato isolates. This, in turns, shows the efficacy of this induced antiserum in recognizing several PVY isolates using DAS-ELISA.

DBIA tests were used successfully for measuring the antigenicity of recombinant CP of PVY with its existing polyclonal antiserum and the induced recombinant antisera (Fig. 6). The recombinant antiserum for PVY was examined for detecting this virus in the field and from known PVY isolates of PVY^{O-N} and -NTN collected locally and from Australian and American (USA) (Fig. 7).

DAS-ELISA and DBIA tests were also used to measure the specificity of the recombinant CP antiserum against local and foreign isolates of PVY. In DAS-ELISA test (Table 1) all isolates tested, except PVY 686 isolate (Australia), reacted positively with the recombinant CP antiserum. However, in DBIA all isolates tested positive with the antiserum including the PVY 686 isolate (Fig. 7). Such results indicate the high selectivity of DAS-ELISA over the indirect ELISA performed on NCM in DBIA. It has been known that indirect ELISA tests are less conserved than DAS-ELISA.

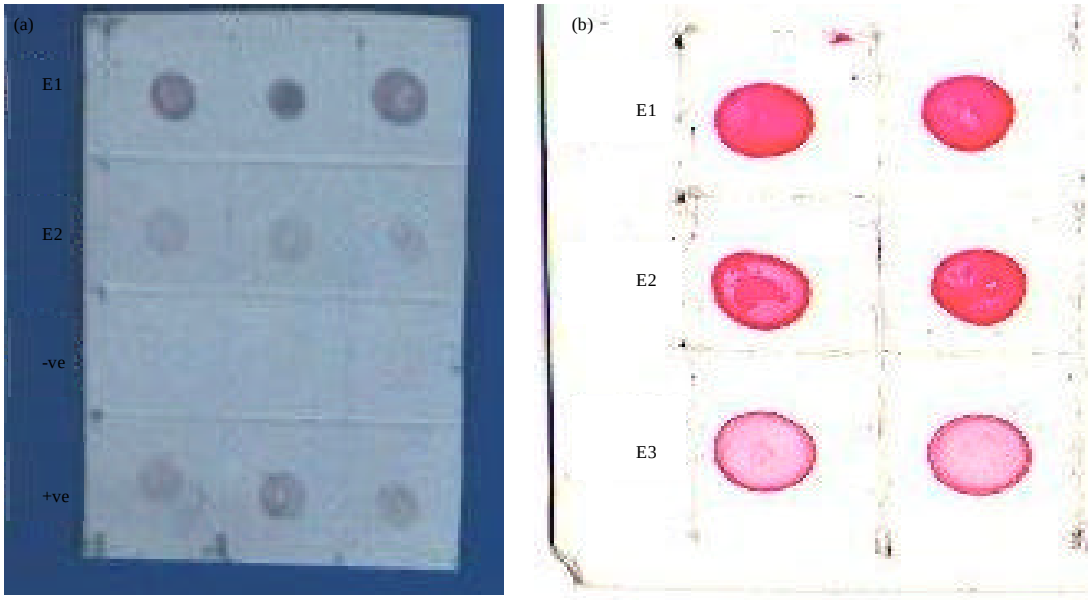

Fig. 6(a-b): Serological evaluation of expressed recombinant PVY CP by DBIA against (a) Its corresponding authentic polyclonal antiserum (P.E. Thomas, Prossor, Washington Stat University, Prossor, USA) and (b) Its corresponding recombinant antiserum. E1 and E2 are 1 μL aliquots of purified recombinant protein and sap of healthy and PVY-infected potato was used as a negative and positive control, respectively. Antiserum was used at 10^{-3} dilutions (a) GAR-AP at 10^{-4} and the blots stained with NBT/BCIP complex for and (b) Naphthol/Fast Red complex for

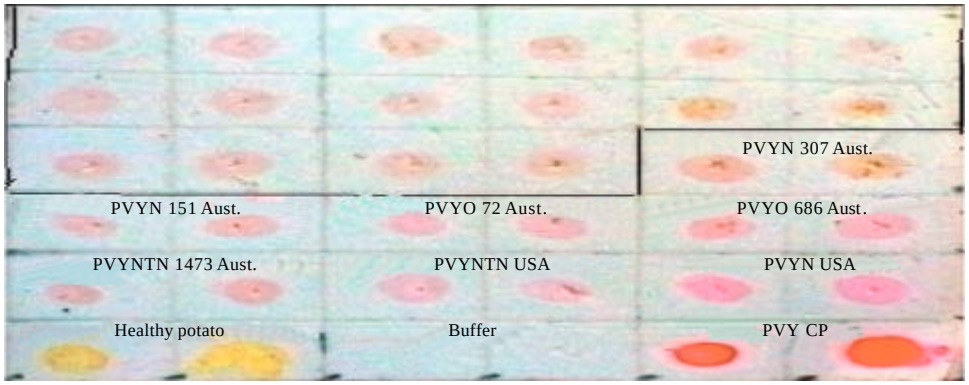

Fig. 7: DBIA evaluation of the specificity of the recombinant PVY CP antiserum against local PVY isolate(s), (marked with black marking pen), PVY^{O}, PVY^{N}, PVY^{NTN} from Australia and the USA. Each sample was repeated twice. The positive control was PVY CP (60 ng μL^{-1}) and the negative control, healthy potato sap and sample coating buffer. All samples were diluted 1:10 (w/v) with coating buffer, pH 9.6. The membrane was stained with Fast Red/Naphthol complex

Table 1: Specificity of recombinant PVY CP antibodies against local and foreign PVY isolates as measured with DAS-ELISA. A positive result was taken as an absorbance (at 405 nm) of 2.5 times the mean of the corresponding healthy control

Isolates of PVY	Origin	Host Plant	*$A_{405\ nm}$
PVY (undefined)	Egypt	Potato	1.17
PVY^{O} 72	Australia**	Potato	1.63
PVY^{O} 686	Australia**	Potato	0.21
PVY^{N} 307	Australia**	Tobacco	0.61
PVY^{N} 151	Australia**	Tomato	0.81
PVY^{NTN} 1473	Australia**	Unknown	1.16
PVY^{N}	USA***	Unknown	0.55
PVY^{NTN}	USA***	Unknown	1.12
Healthy		Potato	0.22
Buffer			0.27

*: Average of two readings, **: Source of isolates, Queensland, Australia, ***: Source of isolates, Beltsville, Maryland, USA

DISCUSSION

The symptoms described in this paper and grouped into four categories generally agree with descriptions made for potato infection with different strains of PVY elsewhere (De Bokx and Huttinga, 1981; Beczner *et al*., 1984; Zitter and Gallenberg, 1984, 1994; Chrzanowska, 1991; Weidemann and Maiss, 1996; Gamal El-Din *et al*., 1997; Oshima *et al*., 2000; Amer *et al*., 2004; Burrows and Zitter, 2005; Kerlan, 2006; Ramirez-Rodriguez *et al*., 2009). From the observed different symptoms developed on potato, it is obvious that more than one PVY isolates or strains are responsible for symptom variations on potato in Egypt.

The purified fusion CP generated in this study measured 32 kDa in SDS-PAGE which is consistent with that reported for PVY-CP by Amer *et al*. (2004) and was antigenically related to known PVY antiserum. However, in evaluating the authenticity of the expressed recombinant protein, two additional smaller bands were detected in both the non-purified and purified fractions

of the expressed CP. These minor bands could represent the expressed V5 epitope and the polyhistidine region in the pBAD-TOPO vector. On the other hand, Watts and Singh (1994) referred to the presence of minor PVY-CP bands associated with the CPs of PVY^{O} and PVY^{N} in immunoblot analysis. The identity of these bands would require further investigation to be certain.

The recombinant PVY-CP antiserum produced in this study was high in specificity for detection of PVY isolates and of expected concentration. When used in IPTA-ELISA. DBIA and DAS-ELISA, the recombinant antiserum successfully detected PVY from several potato varieties. The positive reactivity of our recombinant PVY antiserum in detecting PVY-infected potatoes, using DAS-ELISA, gives a significant value to this antiserum in terms of specificity. A similar recombinant PVY-antiserum was induced by Folwarczna *et al.* (2008). However, this latter antiserum was reactive upon using IPTA-ELISA but not with DAS-ELISA. It is well known that members of the Potyvirus are much conserved in the coat protein domain. Therefore, there would be certain risks in possible involvement of other potyviruses (e.g., *Potato virus A* potyvirus) in infected potato tubers if DAS-ELISA was not available.

Several investigators reported difficulties in using DAS-ELISA for detecting virus antigens upon using recombinant antisera due to the presence of high background in the healthy control (Cerovska *et al.*, 2003; Soliman *et al.*, 2006; Folwarczna *et al.*, 2008). None the less, (Cerovska *et al.*, 2010) successfully used DAS-ELISA in detection of PVX with its recombinant antisera. In the present study, high background problems were detected in the healthy control upon using DAS-ELISA for PVY detection. However, this problem was overcome by adding a blocking step with non-fat dry milk and bovine serum albumin after the IgG coating step. Additionally, many investigators (Cerovska *et al.*, 2003; Folwarczna *et al.*, 2008) indicated the poor reactivity of their recombinant antisera with corresponding native antigens in DAS-ELISA was probably due to CP-antigen alterations during antigen purification under denaturing conditions. For the production of PVY-recombinant antiserum, in the present study, a modified technique of using a mixture of purified PVY-CP, under native and denaturing conditions, to preserve the integrity of internal and external epitopes, was followed. This modification allowed the detection of PVY in potato successfully in DAS-ELISA and WB analysis as well.

CONCLUSION

The present study has shown the production of good quality recombinant antiserum capable of detecting several isolates and strains of PVY infecting potato, tomato and tobacco. This antiserum was initiated from a mixture of recombinant CP purified under native and denaturing conditions to preserve the integrity of internal and external epitopes of PVY-CP. Such modification in the CP purification technique overcome the limitation of using DAS-ELISA upon employing recombinant antisera for virus diagnosis. The induced recombinant PVY antiserum in the present work has shown its potency in detecting most of the PVY strains infecting potato and it should be invaluable in potato quarantines centers worldwide.

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