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Expression of Recombinant Protein of Coat Protein Gene of Sweet Potato Feathery Mottle Virus in Bacterial Expression System and Production of Polyclonal Antiserum for Its Diagnosis

Makeshkumar Thangaraj (makeshctcri@gmail.com)
Central Tuber Crops Research Institute https://orcid.org/0000-0002-3348-2742
Jayakrishnan J T
Central Tuber Crops Research Institute

Research Article

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Abstract

Sweet potato (*Ipomoea batatus* L. (Lam), Family Convolvulaceae) is one of the most important tuber crops providing nutritional security because of its high consumption value and medicinal properties, and numerous agro-industrial uses. Sweet potato feathery mottle disease caused by *Sweet potato feathery mottle virus* (SPFMV) is one of the serious constrains in sweet potato cultivation in India. Effective diagnostic methods need to be developed to solve the problem due to these viruses. As part of the study, infected leaf samples from fields were collected, positive samples were screened for SPFMV using DAC-ELISA and confirmed through PCR. Coat protein gene of SPFMV was PCR amplified, cloned into TA cloning vector and then transformed into *Escherichia coli* DH5α cells. Positive clones were sub cloned into expression vector pET28A(+) and transformed into DH5α cells. Plasmid DNA from positive clones were isolated and transformed into BL21DE3 cells (NiCo21-DE3 cells). Positive clones were identified and confirmed in-frame position through sequence analysis. Selected colony was grown in Luria both medium at 37^{o}C. Cells were collected and solubility of SPFMV coat protein (CP) was checked through SDS PAGE. Various standardisations were carried out for optimising expression of SPFMV CP and it was observed that 4 hr induction of 1.5 mM IPTG at 25^{o}C gives maximum yield. Using these conditions, cells were grown on large scale and purified the protein (SPFMV CP) using Ni-NTA resin affinity chromatography. Purified protein was checked using SDS PAGE, confirmed the expression using Western Blotting and given for immunization into two New Zealand white rabbits for polyclonal antibody production. Serological tests like ELISA and DIBA were done for confirming the sensitivity and specificity of the raised antibody using field samples of SPFMV infected sweet potato along with healthy plants. Tested samples gave strong positive reactions at dilutions of 1:500 up to 1:6000. Also antibody reacted specifically at a dilution of 1:6000 in ELISA and DIBA. This is the first report of development of polyclonal antiserum against CP of SPFMV through recombinant technology in India and can be useful for the detection of virus from the field-grown samples.

Introduction

Root and tuber crops have played an important food reserve especially in areas with marginal ecosystems and unpredictable rainfall. Among the different tropical tuber crops, sweet potato (*Ipomoea batatus* L. (Lam) ranks third after potato and cassava [16]. Worldwide, this crop ranks seventh most important food crop after wheat, rice, maize, potato, barely and cassava [19, 39]. Apart from becoming a rich source of nutrients (carbohydrates, vitamins and minerals), the wide adaptability in any environmental conditions, less input and maintenance cost and high yield made wide acceptance to this crop among the farming communities in the developing countries especially in the tropical regions of the world.

Globally sweet potato is cultivated in 117 countries in the world wherein Africaoccupies first position contributing more than 48% of sweet potato area with a production share of about 20% in total worlds' sweet potato production [32]. Majority of the production of this crop (95%) comes from developing countries in the tropics, of which China have the maximum share (67%) [FAO 2018].

In India, sweet potato is cultivated in almost all the states but major contribution comes from four states namely Odisha, Kerala, West Bengal and Uttar Pradesh [32]. About 1.4 million tones of sweet potato tubers are produced from 1.22 lakh ha area in India [FAO, 2018].

Among the various diseases affecting this crop, Sweet potato feathery mottle disease caused by Sweet potato feathery mottle virus (SPFMV), is the most devastating one affecting sweet potato production worldwide [7, 8, 13, 22, 29, 42] with yield losses ranging from 17 to 30% [13].

This virus belongs to potyviridae family causing more than 70% disease prevalence and with a maximum yield loss of 50% in major sweet potato growing areas in the world [5, 6, 9, 13, 23, 31, 34, 45]. The spread of this dsease is due to the arbitrary use of infected planting material (primary spread) and aphid species, including *Aphis gossypii, Aphis craccivora, Lipaphis erysimi and Myzus perscicae* (secondary spread) [4]. The Symptoms of SPFMV infection depends on the genetic characteristics of the plant and the environmental conditions and it ranges from chlorotic spots, chlorotic vein banding (vein clearing) and irregular chlorotic patterns (feathering) associated with faint or distinct ring spots that have purple pigmented borders [8, 20].

Kumar [28] first reported SPFMV affecting sweet potato in India based on serology and the identity of virus was later confirmed by molecular characterization [18, 20]. Recently, Prasanth [33] reported the occurance of SPFMV in India.

Early diagnostics is essential to eradicate this disease and help preventing the spread to healthy plants. Screening for SPFMV from a large number of samples can be done using symptamatology observation but this technique is not effective since same symptoms can be observed in other infections and the virus can be present in very low titre in symptomless plants also. Therefore, detection using serological methods (involving monoclonal and polyclonal antibodies) got wide acceptance due to the cost and effectiveness for routine detection of a large number of samples. Several serological methods are used worldwide for the detection of SPFMV including Double Antibody Sandwich (DAS) ELISA and Dot ImmunoBinding Assay (DIBA) [1, 5, 12, 16, 20, 27, 40] and Western blotting [44]. Due to the low sensitivity and laborious time consuming assay procedures, serological detection methods have been less effective paving to the development of nucleic acid based technologies for the detection of SPFMV. It has been reported that Nucleic Acid Spot hybridization (NASH) technique can be used for the detection of SPFMV from symptomless plants using strain-specific or wide spectrum non-radioactive or radioactive probes [1, 34]. Polymerisation chain reaction (PCR) and Reverse transcription- Polymerase Chain Reaction (RT-PCR) based methods enable fast and accurate detection of SPFMV [10, 11, 17, 21, 30, 36, 41, 43, 45]. Recently, Kroth [26] developed an immune-capture RT-PCR method of diagnosis of SPFMV. Similarly, a multiplex RT-PCR assay was developed by Rukarwa [35] to detect SPMFV infecting sweet potato plants in Uganda.

More recently, Babu [2] developed a rapid and sensitive detection method combining RT-PCR and NASH techniques for the detection of all the major potyviruses infecting tuber crops (SPFMV in sweet potato, Cassava brown streak virus (CBSV) in cassava, Dioscorea alata virus (DAV) and yam mosaic virus (YMV) in yams and Dasheen mosaic virus (DsMV) in edible aroids).

Though all of these nucleic acid based methods are much accurate and specific, high cost and lack of practicality makes it inappropriate for routine detection with a large number of samples. Therefore, a detection method which is of low cost (affordable to public, especially farming community), specific, sensitive and more suitable for assessing a large number of samples need to be developed for early and quick identification of this virus to device suitable management strategies.

Serological approaches using polyclonal antibodies prepared against recombinant coat protein is commonly used nowadays for the detection and diagnosis of plant viruses [24, 25]. This method utilizes bacterial expression systems for the production of coat proteins from plant viruses and used them as immunogens for the generation of antibodies [14]. The expression and purification of recombinant protein improves appreciably with the additional fusion tag to facilitate the purification, solubility, and stability of the protein product. This purified expressed plant viral protein as a recombinant fusion protein can be used as an antigen for raising virus-specific antibodies for immunodiagnostic purposes. In this point of view, this study aimed at the optimization of expression, production and purification of recombinant coat protein of Sweet potato feathery mottle virus which can be used as antigen for polyclonal antibody production and further the developed polyclonal antibody can be used for the detection of SPFMV in *Ipomoea batatus* L (Lam) in India.

Materials And Methods

Sample collection and screening of SPFMV through serological assays

Leaf samples of sweet potato plants showing characteristic feathering and ring spot symptoms were collected from the field of ICAR- Central Tuber Crops Research Institute (ICAR-CTCRI) (Fig 1).

Preliminary screening was done in order to find out the presence of Sweet potato feathery mottle virus in these samples using both the serological methods like DAC-ELISA and DIBA using antibodies obtained from DSMZ, Germany. Samples which were highly positive for DAC-ELISA and DIBA were taken for subsequent molecular analysis.

RNA isolation

For molecular analysis, total RNA was isolated from the DAC-ELISA and DIBA positive plant leaves by Trizol method (TRI reagent protocol, Sigma-aldrich). Fresh Leaf samples (100 mg) were chilled and pulverized to a fine powder in liquid nitrogen using chilled mortar and pestle and mixed well by adding 1ml TRI reagent (cat#T9424, Sigma-aldrich). The homogenate was transferred to a sterile 2 ml centrifuge tube. To this, 200µl of chloroform was added, mixed well by gentle inversion for 10-30 sec and incubate for 15 min on ice. Then it was centrifuged at 20,000g for 15 min at 4°C. The supernatant was transferred to a fresh tube and extracted twice with an equal volume of 25: 24: 1 (v/v) phenol/chloroform/isoamyl alcohol and mixed well by inversion for 2-3 min. Then it was centrifuged at 20,000g for 10 min at 4°C. To the aqueous phase, 1 ml chilled isopropanol was added and mixed by inversion. The mixture was then incubated at -20^{O}C for 2 hr to precipitate the RNA. The precipitated RNA was pelletized by centrifugation

at 20,000g for 15 min at 4°C. The supernatant was decanted and the pellet was washed twice in 0.5 ml ethanol (70 %) and centrifuged at 20,000g for 5 min at 4°C. Supernatant was discarded and the pellet was air dried for 30-40 min and dissolved in 50 µl deionised water (Incubation at 55^{o}C for 10 min) followed by storage in a deep freezer (-80°C) for later use. Isolated RNA was used as a template for cDNA synthesis using a first strand cDNA synthesis kit according to the manufacturer's instructions (Bioline, Memphis).

PCR amplification and cloning

The cDNA was used as a template to amplify the full-length coat protein gene of SPFMV using the forward primer 5'-GCGGGATCCTCTAGTGAACGTACTGAA -3'(BamHI enzyme site was underlined) and reverse primer 5'-AAAGAGCTCTTGCACACCCCTCATTCC -3' (SacI enzyme site was underlined) to produce an amplicon of 963 bp in length. Reaction mixture per tube were prepared each containing 2.5µl of 10x Taq polymerase buffer (containing 100 mM Tris HCl, 500 mM KCl and 15 mM $MgCl_2$), 0.5µl of dNTPs (10 mM), 0.5µl each of forward and reverse primers, 0.25 µl (2.5 units) of Taq DNA polymerase and 2 µl of template DNA (100 ng) and 13.75 µl of sterile distilled water to make a final volume of 20 µl/tube. The negative controls were the buffer used for RNA extraction and RNA from healthy leaves. The thermal cycling profile was 5 min of initial denaturation at 94°C followed by 35 cycles of: 1 min at 94°C, 45 sec at 57.7^{o}C, 1 min at 72^{o}C and 7 min of final extension at 72^{o}C. Reaction was carried out in DNA BioRad C1000 Touch Thermocycler (Germany). The PCR products (963 bp) were separated by electrophoresis in 1% agarose gel having ethidium bromide as stain (0.5µg/ml) at 70V for about 1hour in 1X- Tris-Acetate–EDTA (TAE) buffer of pH 8.0. The PCR product (963 bp) was excised from the agarose gel using a sharp, disposable blade, put into an Eppendorf tube, and continued with purification using Qiagen gel extraction kit (QIAGEN, United States). The purified amplicon of full-length SPFMV CP was then directly cloned (ligated) into pTZ57R/T (TA cloning vector) using a InsTA clone TA cloning kit (Thermo Fisher Scientific, United States). The ligation product was then transformed into *E. coli* DH5α competent cells using the heat shock method [38]. The transformed white colonies were patched on to a fresh petri plate with Ampicillin (50 µg/ ml final concentration) and LB agar medium containing 40 µl of X- gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (20 mg/ ml in Dimethyl formamide) and 20 µl of IPTG (Isopropyl-β-D-1-thiogalactopyranoside) (0.5 M in sterile water). Selected colonies of bacteria were picked and colony PCR was conducted to determine if the colony contains the DNA fragment or plasmid of interest. The PCR program for the colony PCR was the same as the previous program for the cloning procedure. Putative positive transformants, were selected and plasmid isolation was done by alkali lysis method [37]. Presence of insert was confirmed by restriction with enzymes BamH1 and HindIII. Restriction digestion was performed according to the manufacturer's instructions (New England Biolabs, United States). Positive clones (plasmid) were sent for sequence analysis (Agrigenome labs, Cochin) and confirmed that intact SPFMV coat protein gene is present (PCR amplicon), through NCBI BLASTn program.

Subcloning into expression vector

The Insert (whole coat protein gene CP) was subcloned (ligated) into pET28A(+) expression vector and transformed into DH5α cells [38]. The transformed white colonies (Kanamycin selection) were patched on fresh petri plates with Kanamycin (50 ug/ ml final concentration) and LB agar medium. Confirmation of the insert was done using colony PCR (coat protein gene) and restriction digestion using BamHI. Transformation into pET28A(+) was successful from which putative positive clones were selected through kanamycin selection and plasmid DNA isolated using alkaline lysis method [37]. The positive plasmid (SPFMV-CP in pET28A(+) constructs) was transformed into BL21DE3 cells (NiCo21-DE3 cells cat#C2529H New England Biolabs, United States). Positive clones were identified using restriction digestion with BamH1 and one positive colony from pET28A(+) clones were selected for protein induction and expression studies.

Protein expression and solubility check

Bacterial culture (SPFMV CP positive clone in BL21DE3 cells) was grown in LB medium with Kanamycin (final concentration 50 µg/ml) at 37°C and 200 rpm. After reaching the optical density of 0.8, 1mM concentration of IPTG was added to the bacterial culture and incubated for 3 hrs at 37°C for the IPTG induced production of the recombinant protein (SPFMV CP). Similarly, another set of bacterial culture was grown seperately in LB medium with Kanamycin at 37°C and 200 rpm for 3hrs without IPTG induction and this was used as uninduced control. Cells from both IPTG induced culture and uninduced control were harvested by centrifugation at 20,000g for 10 min at 4°C and the total protein was extracted using lysis buffer (containing 20 mM Tris pH 8, 100mM NaCl, 0.1% Triton-X-100, 1mM EDTA pH 8, 2M Urea and 10X Protease Inhibitory Cocktail (PIC) (cat#G6521, Promega, United States)). Total cell lysate was loaded on 15% acrylamide-bisacrylamide gel and run the SDS PAGE to confirm the protein induction. Western Blotting was conducted to confirm the expression of protein (SPFMV CP) using crude extracts of the sample and the results were analysed through staining with BCIP/NBT solution.

Solubility of the recombinant protein was checked according to the procedures in ‘The QIAexpressionist handbook for high level expression and purification of 6xHis-tagged proteins’ (QIAGEN, United States). Both insoluble and soluble fractions were collected from IPTG induced bacterial culture and SDS PAGE analysis was done to confirm the presence of protein of interest is in which fraction. The same was confirmed through Dot Immunobinding Assay (DIBA).

Optimisation of protein expression

Three different temperatures namely 25^{o}C, 31^{o}C and 37^{o}C, four different concentrations of IPTG namely 0.5 mM, 1mM, 1.5mM and 2mM and five different time points namely 30 min, 1 hr, 2 hr, 3 hr and 4hr were selected for SPFMV CP protein standardization experiments. Using these conditions, bacterial culture (SPFMV CP positive clone in BL21DE3 cells) were grown, induced with IPTG and total protein was extracted. Insoluble fractions were collected from the total protein and SDS PAGE analysis was done in 15% acrylamide-bisacrylamide gel to check the optimum temperature, concentration of IPTG and the desired time point after IPTG induction in which maximum yield of protein is to be obtained.The results

show that an IPTG concentration of 1 mM is giving maximum induction and a time period of 3 hrs after IPTG induction at 37^{O}C gives the maximum yield.

Protein purification and polyclonal antibody production

Using the above stated conditions, cells were grown and induced with IPTG and purified the protein (under denaturing conditions) using Ni-NTA resin affinity chromatography (QIAExpress Type IV kit). SDS PAGE analysis and western blotting were conducted to confirm the expression of purified protein (SPFMV CP). Large scale production and purification of the recombinant protein (SPFMV CP) was carried out using the optimized conditions and the concentration of the purified protein was determined according to Bradford (1976). Purified protein was given to two New Zealand white rabbits for producing polyclonal antibody.

Immunization of rabbits, absorption and purification of antibodies

Immunization of rabbits for polyclonal antibody production and purification were given for outsourcing (Abgenix India Private limited) where the purified recombinant SPFMV CP was used as the antigen to raise antibodies in the two New Zealand White rabbits. First and third bleed serum were collected one and two months respectively after immunization from both rabbits. Indirect ELISA was done using both the bleeds with 1:5000 dilution against custom antigen (purified recombinant SPFMV CP protein) to evaluate the strength of reactivity and the optimal concentration of the antiserum raised.

After completing the sensitivity assay, antisera raised was purified and confirmed the reactivity of the purified antibody through ELISA and western blot analysis.

Optimization and calibration of developed polyclonal antibody

Specificity analysis of polyclonal antibody using DAC-ELISA and DIBA

DAC-ELISA was done using polyclonal antibody (IgG) developed against the recombinant SPFMV coat protein using the standard protocol (Hegde et al. 2010). Crude leaf extracts from SPFMV positive samples and leaf samples showing feathering symptoms were tested along with healthy leaves from aseptically raised sweet potato plants and non-host plants (tissue culture raised cassava plants) (negative control). The result was evaluated after adding universal alkaline phosphatase–conjugated anti-rabbit IgG (Sigma Aldrich, United States) by measuring light absorbance at 405 nm wavelength. The samples were positively identified if the mean DAC-ELISA (Absorbance at 405 nm) value of samples exceeded at least twice the mean of the healthy control.

Crude leaf extracts from SPFMV positive samples and field samples showing feathering symptoms were spotted on nitrocellulose membrane (NCM) and used for DIBA analysis according to the satnderd protocol (Hegde et al. 2010) for analyzing the specificity of purified antibody. The universal alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Aldrich, United States) was used as the secondary

antibody at a dilution of 1: 10,000. The target proteins were finally revealed as purple spotes on NCM by adding to substrate 5-bromo-4-chloro-3- indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT)

Sensitivity analysis of polyclonal antibody using DAC-ELISA

To determine the sensitivity of polyclonal antibody (IgG) developed against the recombinant SPFMV coat protein, DAC-ELISA was done using the standard protocol (Hegde et al. 2010) wherein different dilutions were used for optimizing the reactivity of the IgG (1:100, 1:500, 1:1000, 1:2000, 1:3000, 1:4000, 1:5000, 1:6000, 1:7000, 1:8000, 1:9000 and 1:10000). SPFMV positive samples along with negative control (healthy leaves from aseptically raised sweet potato plants) were tested. The result was evaluated after adding universal alkaline phosphatase–conjugated anti-rabbit IgG (Sigma Aldrich, United States) by measuring light absorbance at 405 nm wavelength.

Results

Detection of Sweet potato feathery mottle virus and cloning of full-length coat protein gene

Based on the DAC-ELISA and DIBA results, highly positive samples were selected and PCR amplification was performed using the total RNA and full-length SPFMV CP primers. Primers were designed by multiple alignment of the full-length CP gene from several SPFMV isolates from National Centre for Biotechnology Information (NCBI) database (Table 1). The nucleotide sequence was adjusted between the recognition site of the restriction enzymes, BamHI and SacI, in pET-28a (+) vector and the conserved regions of the CP gene so that it did not change nucleotide sequence encoding the protein and retained the nucleotide sequence in the expression vector. This produced an amplicon fragment with a size of ~1000bp and this SPFMV CP gene fragment was cloned into vector pTZ57R/T (TA cloning vector) and transformed into *E. coli* DH5α cells. The purpose of cloning to this plasmid was to store the targeted DNA fragment in a cloning vector having a T-end and also to perform the sequencing of the PCR product thereby confirming the presence of intact SPFMV CP. Plasmid DNA containing the putative recombinant plasmid (pTZ57R/T with SPFMV CP) was isolated from white colonies of bacteria grown in selection media. A total of six bacterial colonies were analyzed and verified that only one colony carries the insert with a size of approximately 1000 bp DNA (Fig 2). Plasmid DNA from all these six samples were digested with BamHI and HindIII to confirm the release of insert (Fig 3) and given for sequencing.

Sequence analysis showed that Intact SPFMV CP sequence was present in the clones (recombinant plasmid (pTZ57R/T with SPFMV CP) with 963 bp length. Nucleotide and amino acid sequence comparisons showed the highest homology with SPFMV Korean isolate (NCBI ID KP115608.1). The SPFMV CP gene that was cloned in TA cloning vector was cut along with the expression vector pET-28a (+) with two restriction enzymes (BamHI and SacI) to obtain a cohesive end and transformed into *E. coli* DH5α cells. The BamHI and SacI restriction enzymes will only cut on the multiple cloning sites (MCS) and will fuse with histidine at both ends (6x polyhistidine tag). MCS truncated parts will be replaced by the SPFMV CP gene. A total of nine colonies from the selection media were analysed using PCR with SPFMV CP specific primers (Colony PCR) and confirmed seven of these nine colonies as positive (Fig 4). These

colonies were reconfirmed again using restriction digestion of plasmid DNA with BamHI (Fig 5), wherein colony four was selected and the plasmid DNA was transformed into BL21DE3 cells (cells for protein expression). Four transformed colonies were selected, confirmed with restriction digestion using BamHI (Fig 6) and colony 2 was selected for the expression studies.

Expression and purification of Sweet potato feathery mottle virus coat protein

The expression of SPFMV CP protein was induced by 1 mM IPTG induction for 3 hrs at 37^{o}C and the same was confirmed using SDS PAGE analysis (Fig 7). Solubility of the protein was analysed using SDS PAGE and confirmed that the protein comes in insoluble fraction (Fig 8).

Among the three different temperatures used for inducing the protein namely 25^{o}C, 31^{o}C and 37^{o}C, it was found that maximum induction of SPFMV CP occurs at 25^{o}C (Fig 9).

Among the four different concentrations of IPTG used for inducing the protein namely 0.5 mM, 1mM, 1.5mM and 2mM, it was found that concentration of 1.5 mM is giving maximum induction (Fig 10).

Among the five different time points selected for the study, namely 30 min, 1 hr, 2 hr, 3 hr and 4hr after IPTG induction, it was found that time period of 3 hrs after IPTG induction gives the maximum yield (Fig 11). The protein band exhibited significant changes in terms of thickness at all levels of induction; however, higher concentrations of IPTG were found to be suitable for high levels of expression. The optimum concentration of IPTG at 1.5 mM for 3 hr at 25^{o}C was used for over-expression of SPFMV CP protein.

The results of SDS PAGE showed that the recombinant protein was expressed with a molecular weight of about 41 kDa. The inserted conserved region of the SPFMV coat protein gene was located in frame with the sequence encoding six histidine residues that function as a metal binding domain in protein translation. Their N-terminal fusion peptide in the vector will add 4-5 kDa protein expressed. Thus, the protein molecular weight expression resulting from the recombinant construct of SPFMV CP was approximately 41 kDa (Fig 7).

The over expressed protein was produced in large scale by IPTG induction and purified using Ni-NTA column chromatography (QIAexpression type IV kit). Purified elutes (elute C, D and E) were analysed on SDS PAGE (Fig 12) and western blotting was conducted to confirm the expression of purified protein (different elutes) (Fig 13). It was found that elutes D and E were the required expressed protein. These elutes were pooled together for making the concentration of protein optimum for antibody production when being injected into the animal sample. Concentration of the expressed protein (pooled elutes) were determined using Bradford assay and was found to be 0.455g/ml. According to the Institutional Animal Care and Use Committee (2016), the optimum concentration of antigen for antibody induction is 50-1000 µg for rabbits and 10-200 µg for mice. So, 5 ml of purified elute was given for immunization into two New Zealand white rabbits (Abgenix Pvt ltd, India).

SPFMV Polyclonal antibody production and optimization

Purified SPFMV CP was given for immunization into two New Zealand White rabbits (Abgenix India Private Limited). After the primary immunization, Indirect ELISA done to check the reactivity of the antisera wherein the collected sera (first and third bleed) were tested against SPFMV purified CP antigen at dilution of 1: 5000. After 15 min of enzyme substrate reaction, absorbance value of 2.043 and 2.136 were obtained by first bleed of rabbit A and rabbit B respectively wherein an absorbance value of 2.228 and 2.424 were obtained from third bleed of rabbit A and rabbit B respectively. Further, purified polyclonal antisera were evaluated using indirect ELISA (Fig. 14) and western blotting and the results were promising. An absorbance value of 2.324 at 405nm was obtained when tested against custom antigen (recombinant SPFMV CP). The result showed that the antisera reacted well with the custom antigen protein and gave a clear dark band with a molecular size of 41 kDa (Fig. 15). The company provided the purified IgG in liquid for with a concentration of 6.56 mg/ml.

Results from DAC-ELISA and DIBA for checking the specificity of the raised polyclonal antibody revealed that the developed IgG reacted very efficiently with the SPFMV infected tissue and gave promising result. However no signal was observed with the crude extracts from healthy plants. With the ten samples tested (including healthy leaves and buffer control) with DAC-ELISA, four samples show highly positive absorbance value at 405 nm. Compared with symptomatic leaves, absorbance value of the crude extract from healthy leaves and the buffer control were lower (Fig. 16). DIBA analysis result was further more specific than DAC-ELISA since the result was more observable as purple spots. With the nine field samples tested, three samples got high intensity purple colour spot (Fig) wherein healthy sweet potato samples, non-host samples (cassava) and buffer control gave no colour indicating the high specificity of the raised antibody (Fig. 17)

Results from DAC-ELISA which was performed to evaluate the sensitivity of raised polyclonal antibody using different dilutions revealed that the developed IgG reacted very efficiently with SPFMV positive samples and gave positive results upto 1:6000 dilutions (Fig. 18)

Discussion

In this study, we successfully cloned the SPFMV CP gene obtained from SPFMV infected sweet potato leaves and efficiently expressed in a bacterial expression system (pET28A(+)), and this was used as an antigen for the production of polyclonal antibodies against Sweet potato feathery mottle virus. The cloning strategy used for construction of the SPFMV CP protein resulted in the addition of 4-5 kDa of the N-terminus. Thus, the molecular weight of the protein expression resulting from recombinant SPFMV CP was approximately 41 kDa. Hence, the coat protein was expressed as a fusion protein with an estimated molecular weight of about 41 kDa, which is about 6 kDa more than the expected molecular weight of 35 kDa. Based on these results, recombinant SPFMV CP was successfully produced and expressed in the bacterial expression system. Furthermore, SPFMV CP in highly purified, specific, and high concentration form was obtained with the use of a Ni-NTA resin column purification technique, thereby we could readily

gather large amount of proteins using this method (0.45 mg/ml), which is sufficient to raise antibodies. Thus, the SPFMV CP protein obtained from this study was suitable as a proper antigen and was given for immunization into two New Zealand white rabbits for the production of polyclonal antibody. Antibody thus produced was tested in DAC-ELISA and DIBA with field samples of SPFMV infected sweet potato along with healthy plants. Tested samples gave strong positive reactions at antibody dilutions of 1:500 up to 1:6000. The antiserum raised against recombinant CP protein reacted specifically at a dilution of 1:6000 in DAC ELISA and DIBA.

Detection and diagnosis of viruses through molecular assays such as PCR, RT-PCR and sequencing is costly, time-consuming as well as requires sophisticated equipments and trained personnel. In contrast, detection of viruses through serological assays such as ELISA, DIBA and immunostrips using polyclonal antibody is cheaper and less time-consuming without require any expertise or high cost instruments. The present study revealed that the recombinant antiserum produced against the coat protein (CP) of SPFMV can serve as a potential serological diagnostic tool for the detection of SPFMV in India. Immunostrips (Antibody coated strips) are widely utilized in the diagnosis of on-farm viral diseases since these are one of the fastest serological tests easily available in the market. Antiserum raised in this study can be utilized for the development of immunostrips to detect SPFMV. This is the first report of development of polyclonal antiserum against CP of SPFMV through recombinant technology in India and can be useful for the detection of virus from the field-grown samples.

Declarations

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Code availability

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Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval - Not applicable

Consent to participate

Consent for publication - Yes

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Tables

Table.1 List of SPFMV isolates available in NCBI which were analyzed for the primer synthesis

NCBI sequence ID	Sequence description
MG65642.1	Sweet potato feathery mottle virus isolate Aus13-18B-1
NC_001841.1	Sweet potato feathery mottle virus complete gene
MF185715.1	Sweet potato feathery mottle virus isolate SPFMV-UNB-01
KY296450.1	Sweet potato feathery mottle virus strain O
KY296451.1	Sweet potato feathery mottle virus strain RC
D86371.1	Sweet potato feathery mottle virus genomic RNA
FJ155666.1	Sweet potato feathery mottle virus isolate Piu3
KP115610.1	Sweet potato feathery mottle virus isolate IS90
KP115609.1	Sweet potato feathery mottle virus isolate GJ122
KP115608.1	Sweet potato feathery mottle virus isolate CW137

Figures

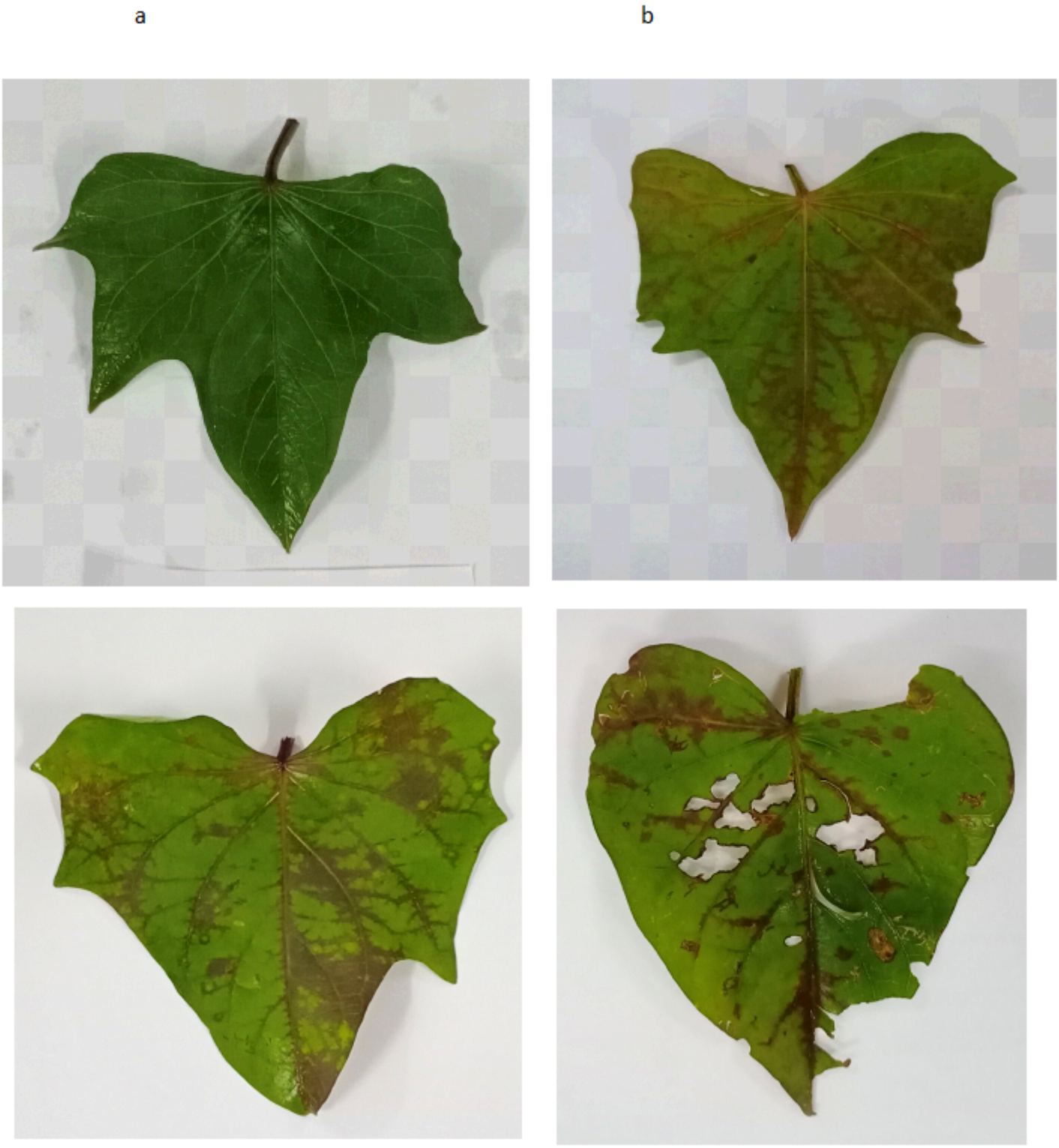

Figure 1

Sweet potato healthy leaf (A) and infected leaf (B) showing typical feathering symptoms of Sweet potato feathery mottle virus Sweet potato leaves collected during the current study exhibiting typical feathering symptoms of Sweet potato feathery mottle virus

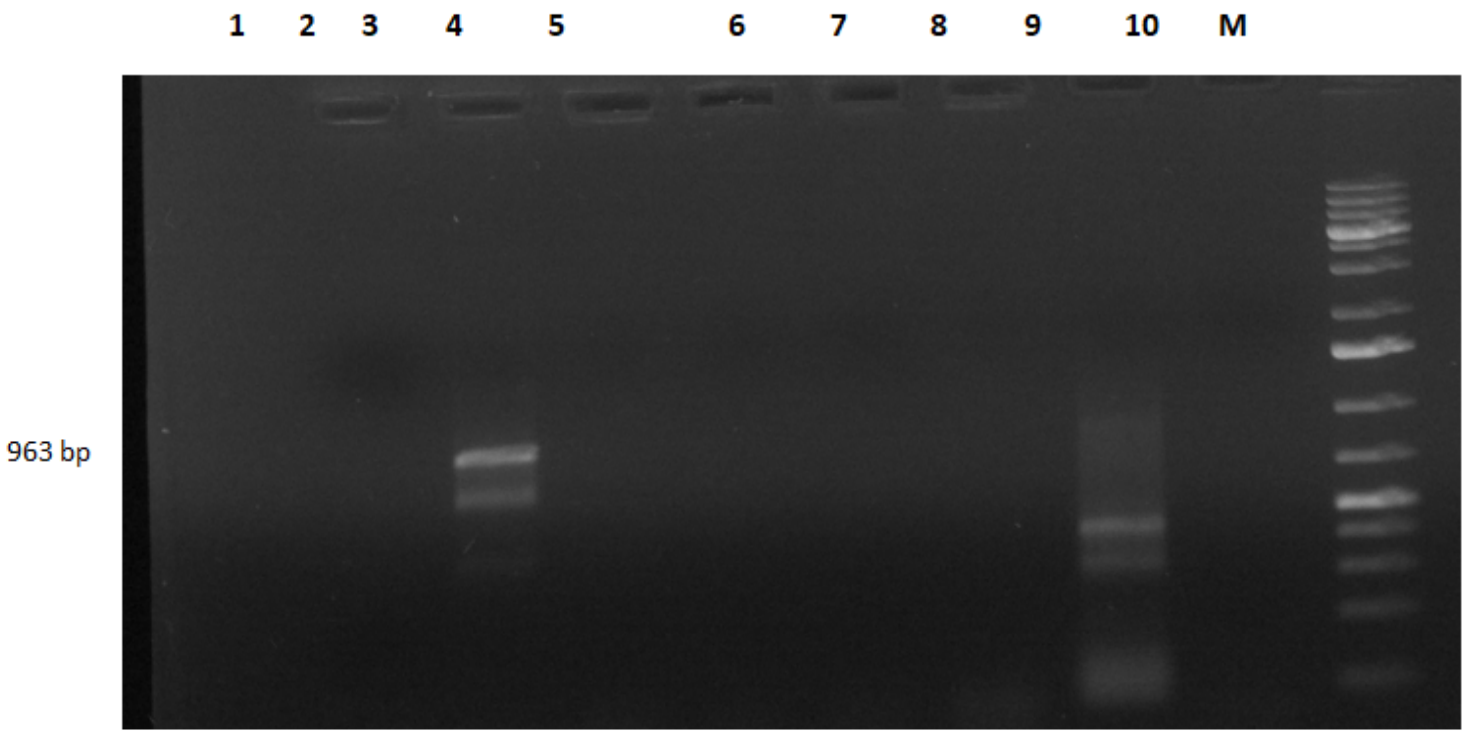

Figure 2

Gel image of colony PCR for SPFMV coat protein gene after cloning with TA cloning vector and transformed into E. coli DH5α strain. M, marker 1 kbplus; Lanes 1-10, white colonies; lane 11, negative control. Numbers at sides indicate the sizes of marker fragments in base pairs

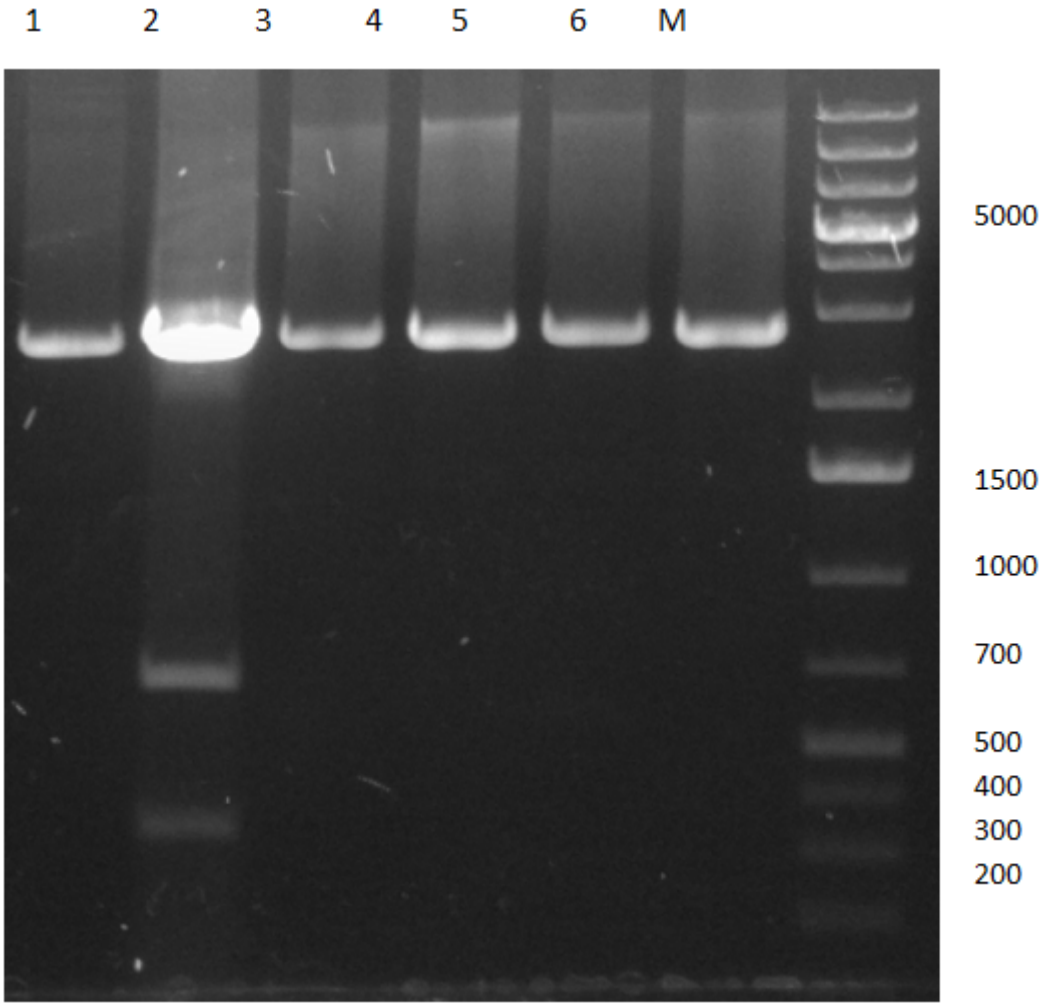

Figure 3

Restriction digestion of the plasmids. M, marker 1 kplus;Lanes 1-6, plasmid DNA from white colonies digested with BamHI and HindIII. Numbers at sides indicate the sizes of marker fragments in base pairs

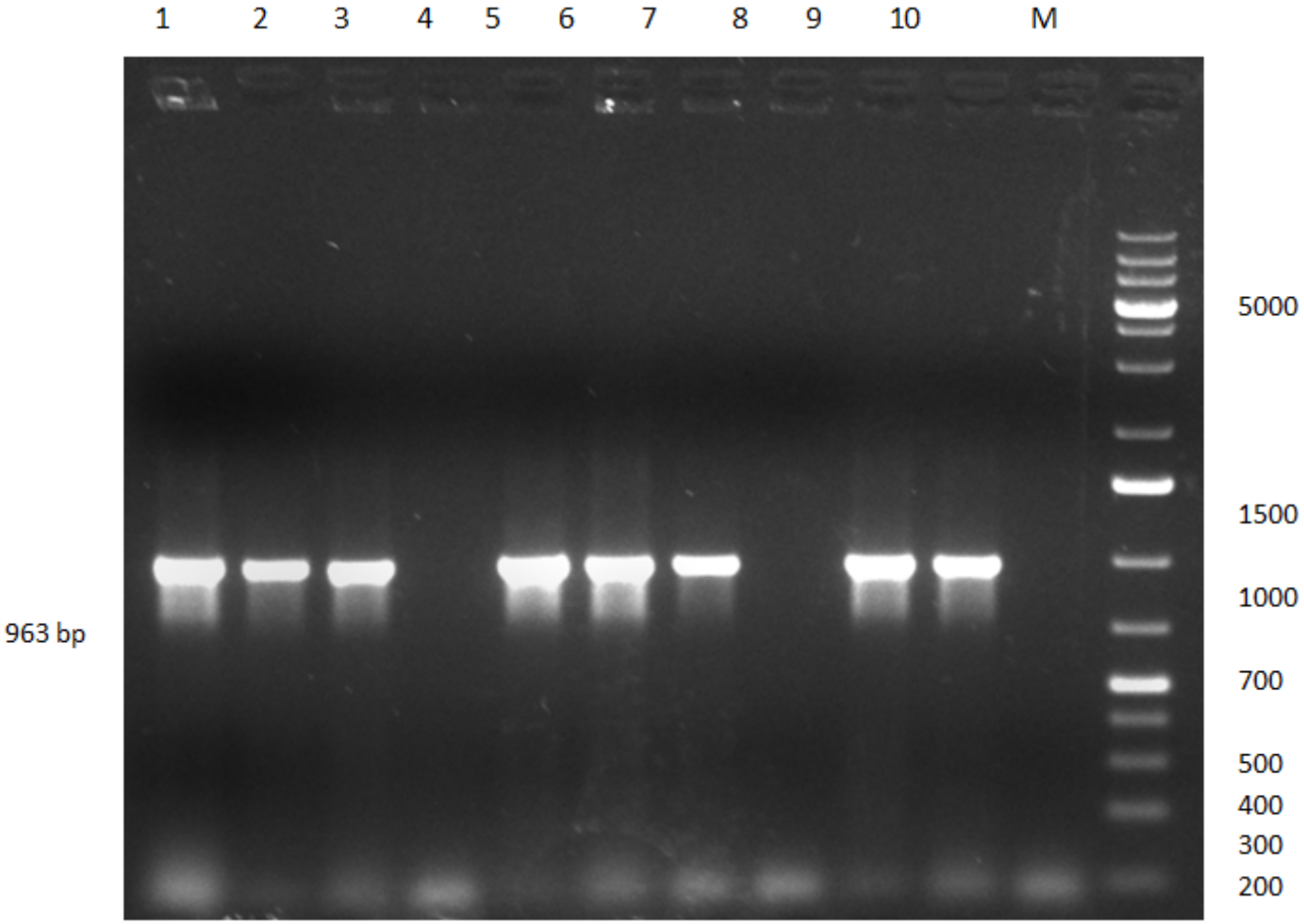

Figure 4

Colony PCR for coat protein gene after cloning with pET28A+ vector and transformed into E. coli DH5α strain. M, marker 1 kplus;Lanes 1-9, white colonies obtained after transformation; lane 10, positive control; lane 11, negative control. Numbers at sides indicate the sizes of marker fragments in base pairs

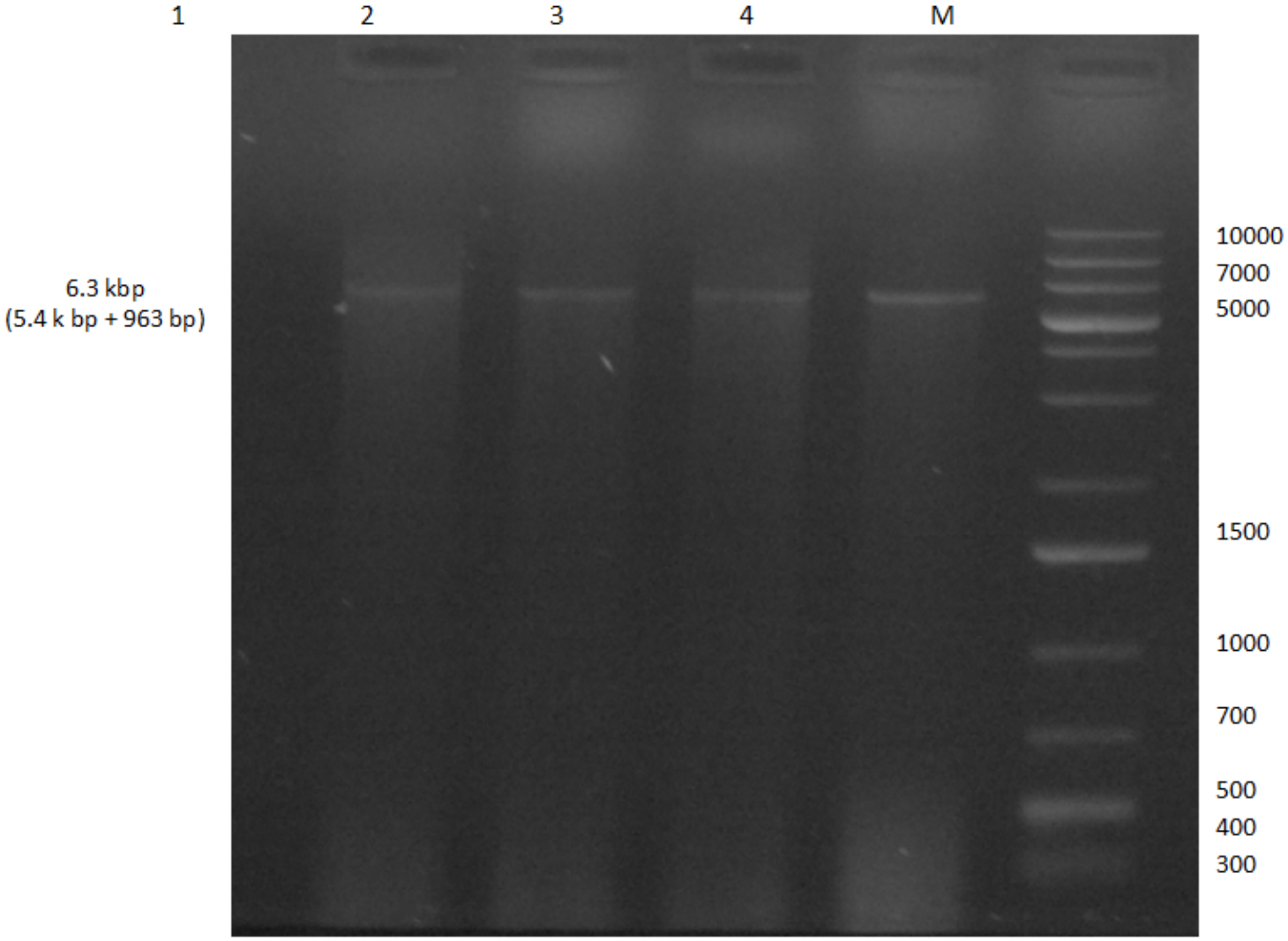

Figure 5

Restriction digestion of the plasmids with BamH1. M, marker 1 kbplus;Lanes 1- 4, colony PCR positive samples. Numbers at sides indicate the sizes of marker fragments in base pairs

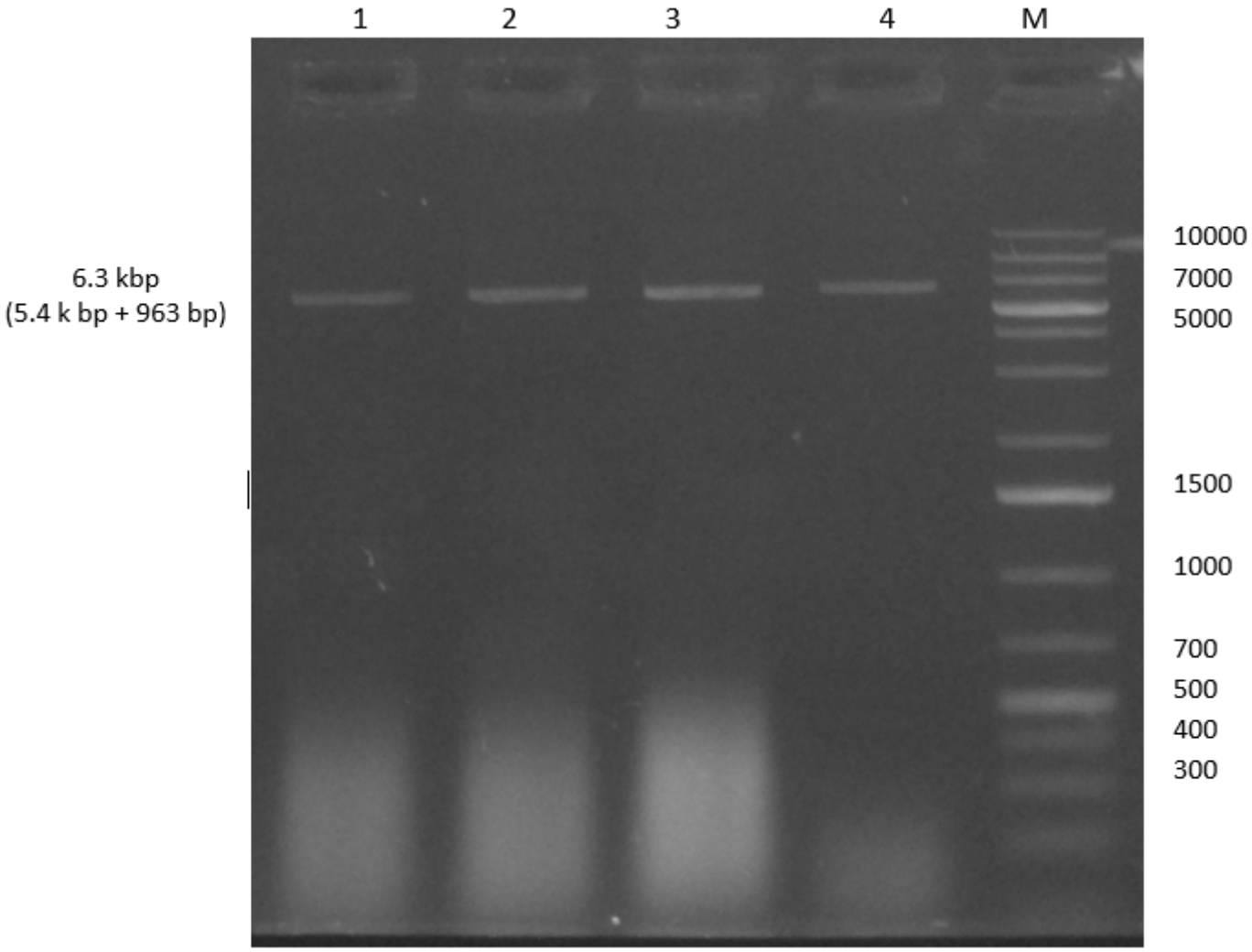

Figure 6

Restriction digestion of the plasmids with BamHI, isolated from white colonies obtained after Transformation of SPFMV-CP construct in E. coli BL21 DE3 strain. M, marker 1 kbplus;Lanes 1-4, plasmid DNA isolated from white colonies obtained after transformation. Numbers at sides indicate the sizes of marker fragments in base pairs

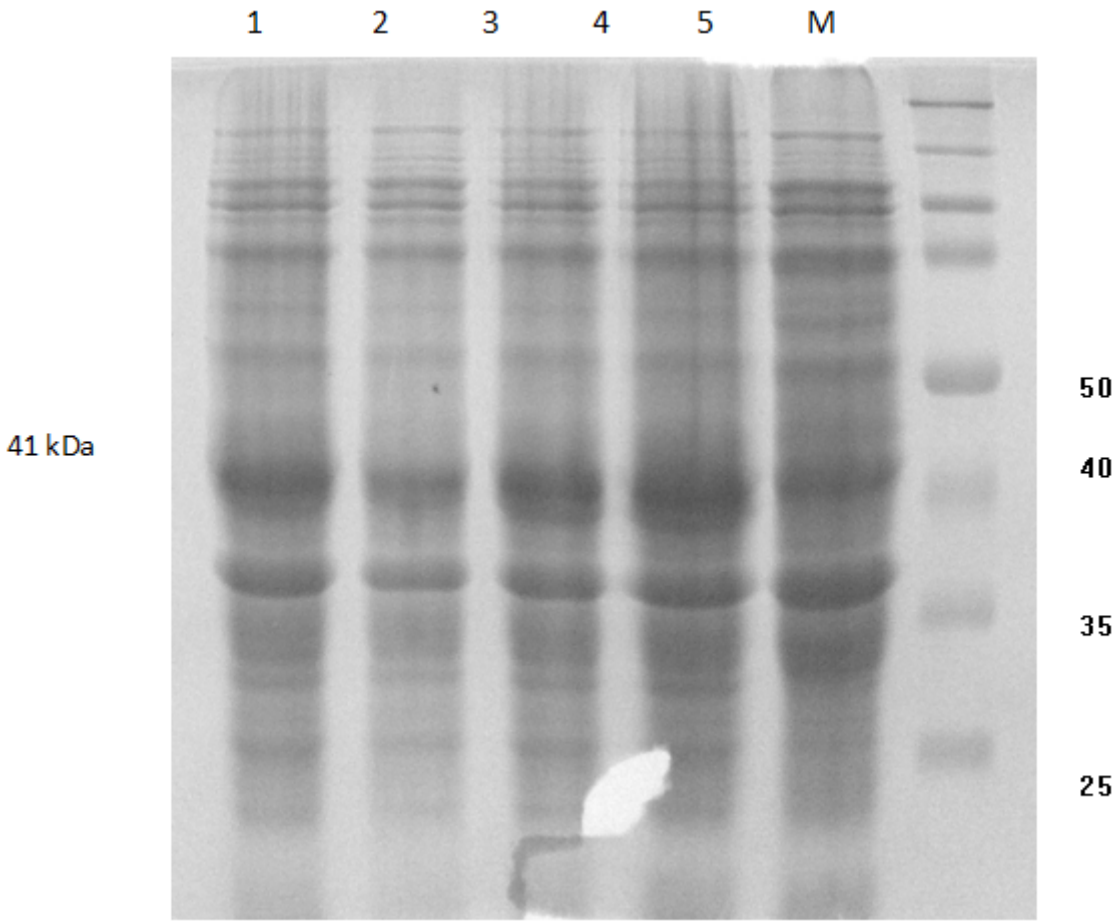

Figure 7

Analysis of crude protein fractions of SPFMV CP (1 mM IPTG induced) in E. coli BL21 DE3 strain by SDS PAGE. M, marker prestained broad range; Lanes 1-4, SPFMV CP-pET28A+ construct in BL21 DE3 strain colonies; lane 5, uninduced control. Numbers at sides indicate the sizes of marker fragments in kilo daltons

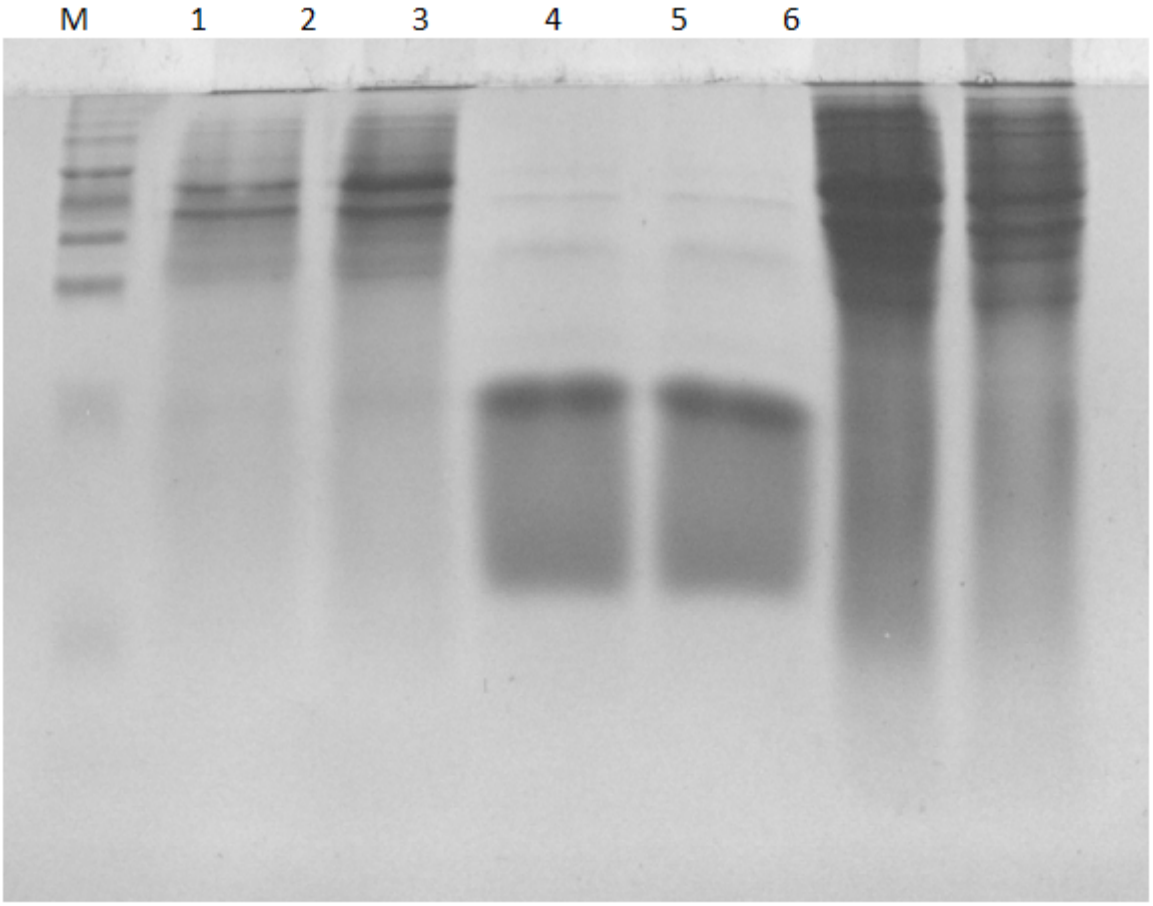

Figure 8

Analysis with soluble and insoluble fractions of crude protein to check the presence of SPFMV coat protein. M, marker prestained broad range; Lanes 1-2, insoluble fraction; lanes 3-4, soluble fraction; lane 5-induced control; lane 6-uninduced control. Numbers at sides indicate the sizes of marker fragments in kilo daltons

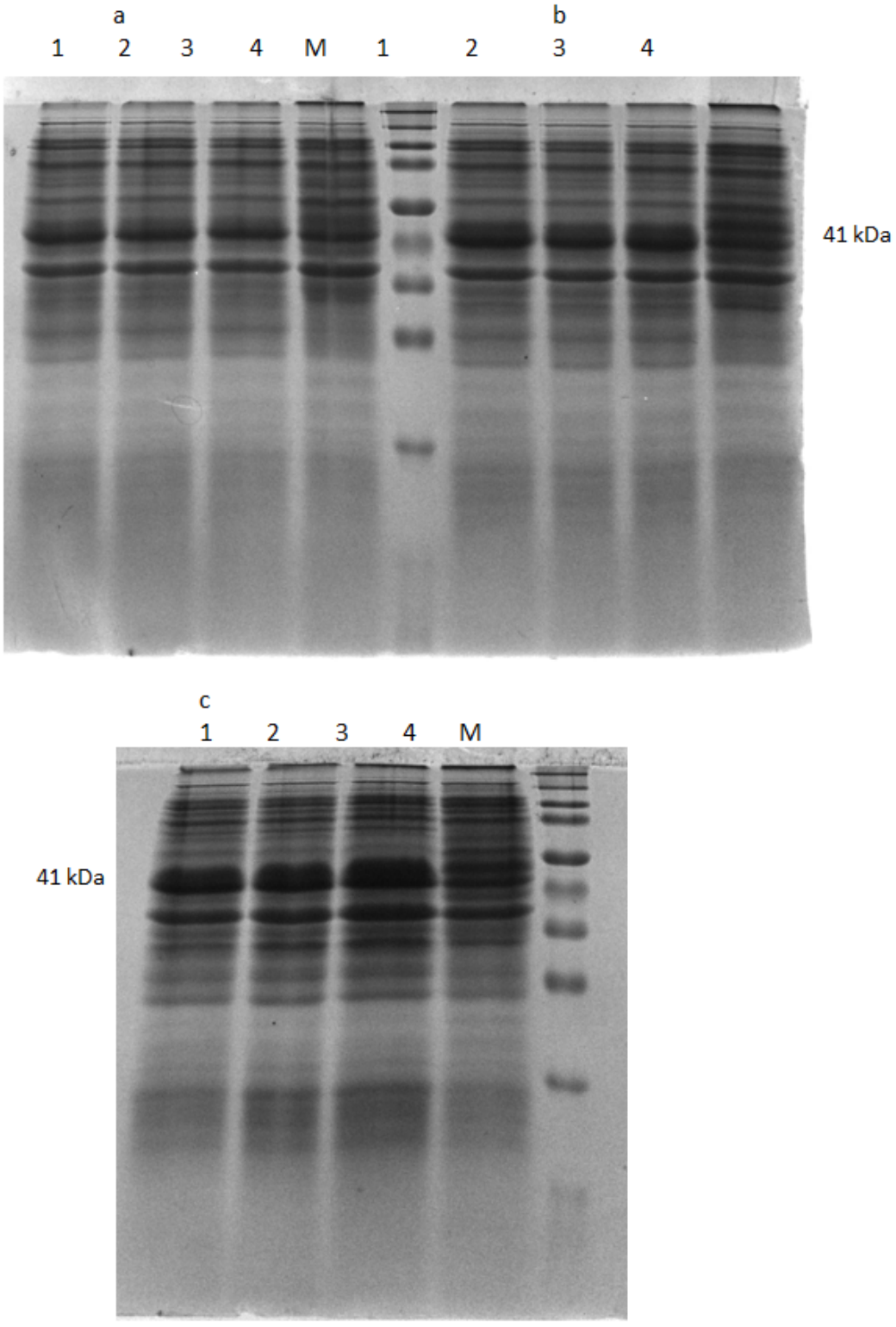

Figure 9

Analysis of total proteins extracted from E. coli BL21 DE3 strain by SDS-PAGE (Temperature standardisation). Induction temperatures 37oC (A), 31oC (B) and 25oC (C); M, marker prestained broad range; Lanes 1-3, induced samples; lane 4, uninduced control

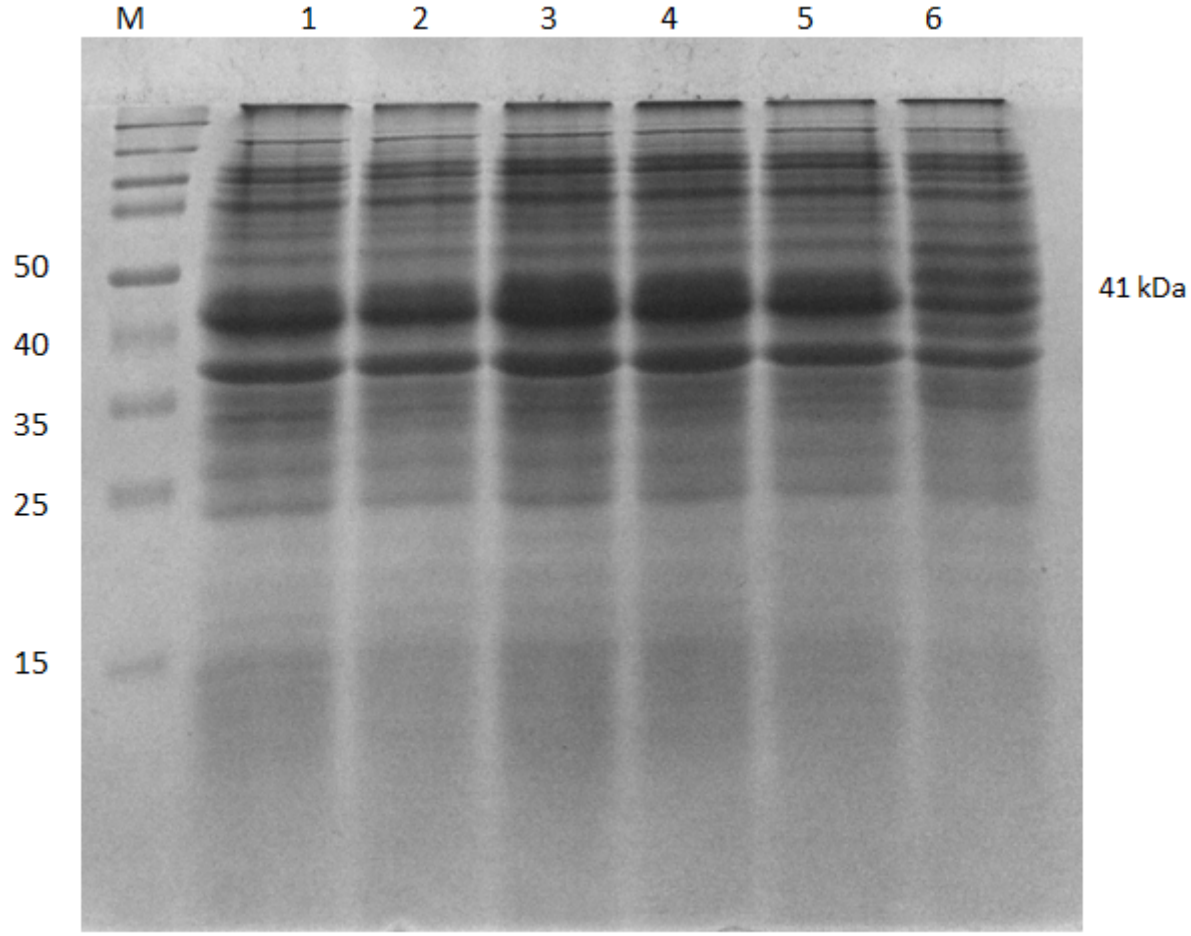

Figure 10

Analysis of total proteins extracted from E. coli BL21 DE3 strain by SDS-PAGE (IPTG standardisation, Induction temperature- 25oC). M, marker prestained broad range;Lane 1, samples collected after induction with 0.5 mM IPTG; lane 2, samples collected after induction with 1 mM IPTG; lane 3, samples collected after induction with 1.5 mM IPTG; lane 4, samples collected after induction with 2 mM IPTG; lane 5, samples collected after induction with 2.5 mM IPTG; lane 6, samples collected without induction. Numbers at sides indicate the sizes of marker fragments in kilo daltons

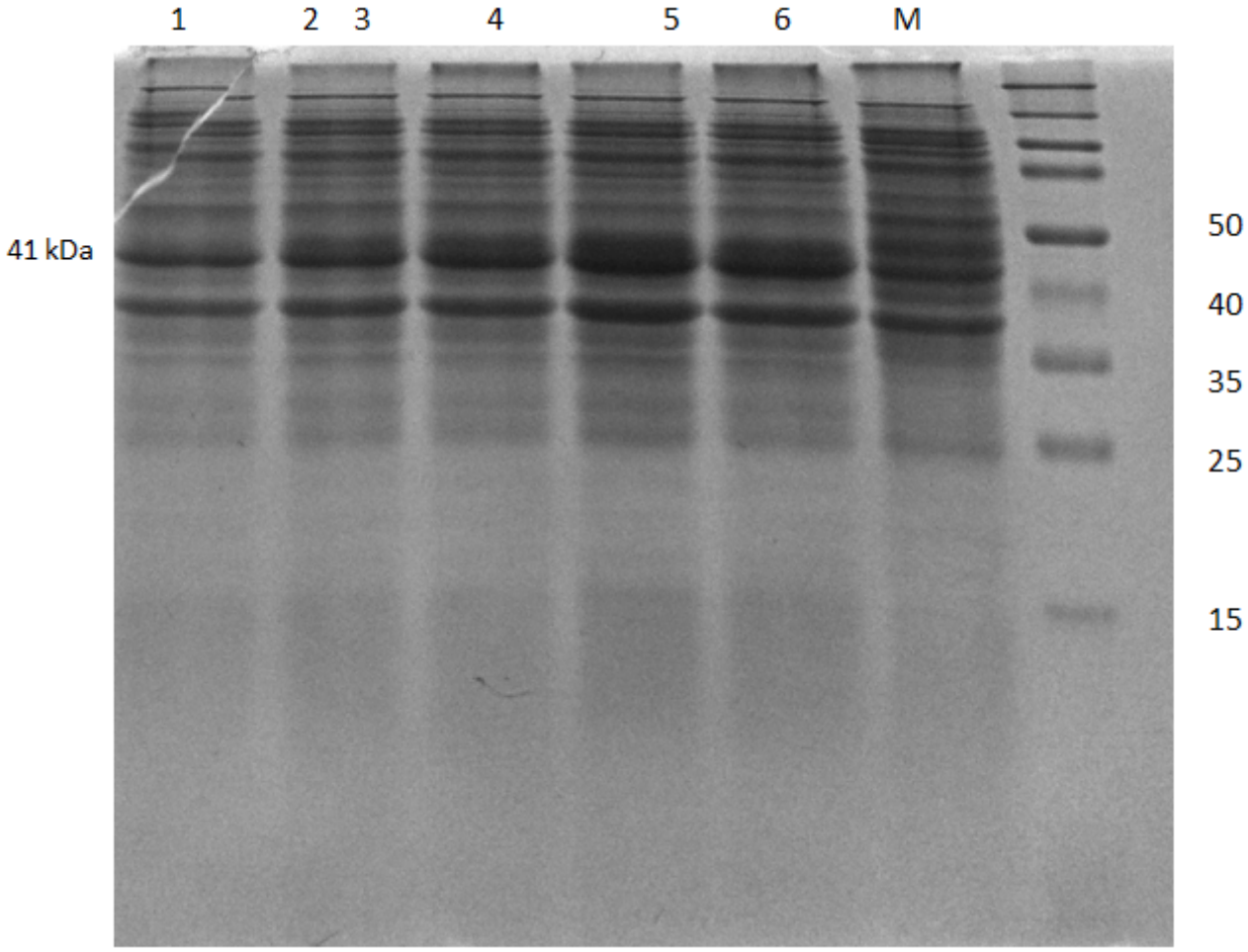

Figure 11

Analysis of total proteins extracted from E. coli BL21 DE3 strain by SDS-PAGE (Time standardisation, Induction temperature- 25oC, IPTG concentration- 1.5 mM). M, marker prestained broad range;Lane 1, samples collected after 30 min; lane 2, samples collected after 1 hr; lane 3, samples collected after 2 hr; lane 4, samples collected after 3 hr; lane 5, samples collected after 4 hr; lane 6, samples collected without induction. Numbers at sides indicate the sizes of marker fragments in kilo daltons

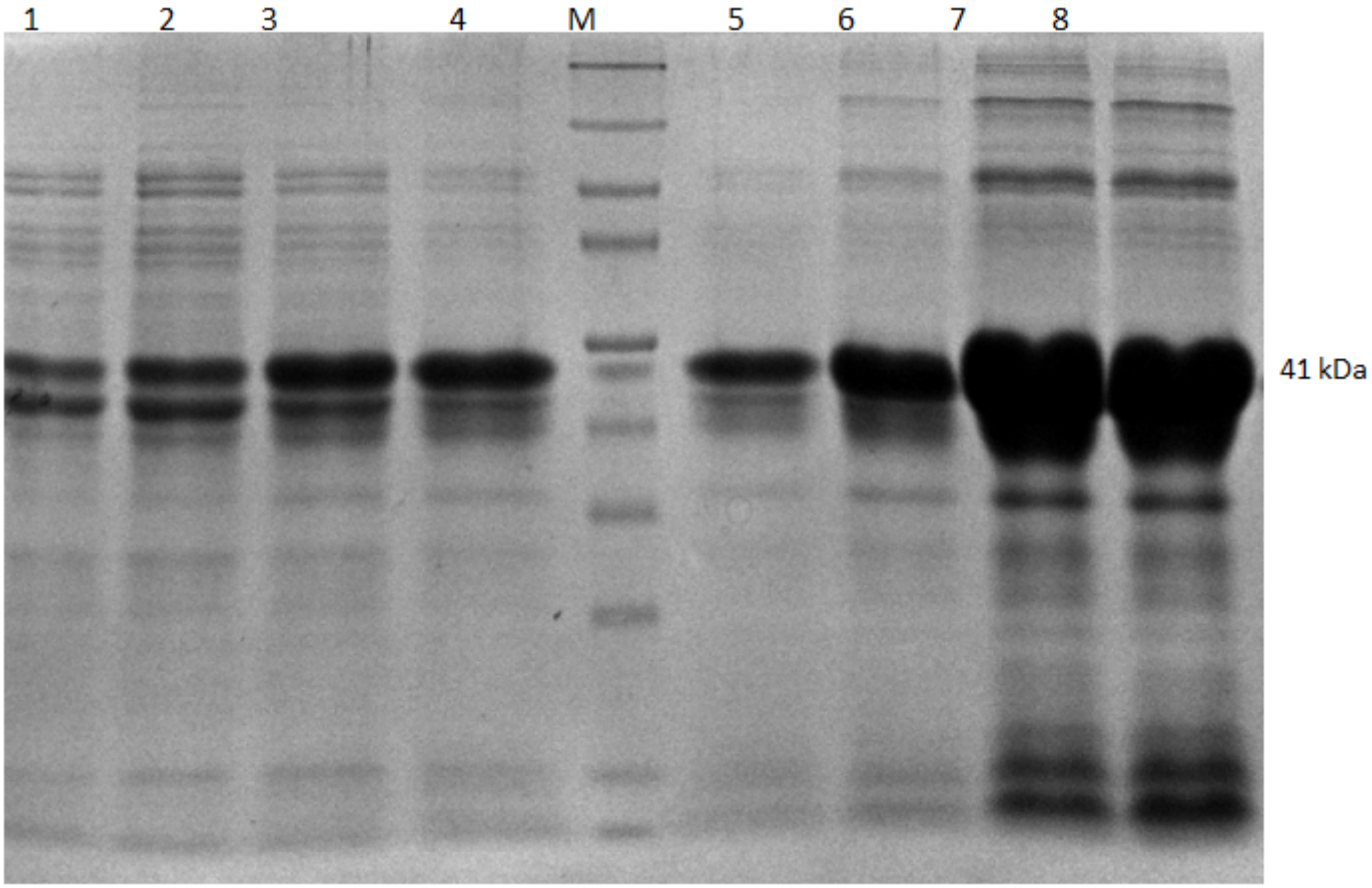

Figure 12

Analysis of purified elutes extracted from recombinant E. coli BL21 strains by SDS-PAGE. M, marker prestained broad range; Lanes 1-4, SPFMV-CP protein eluted using buffer D; lanes 5-8, SPFMV-CP protein eluted using buffer E.

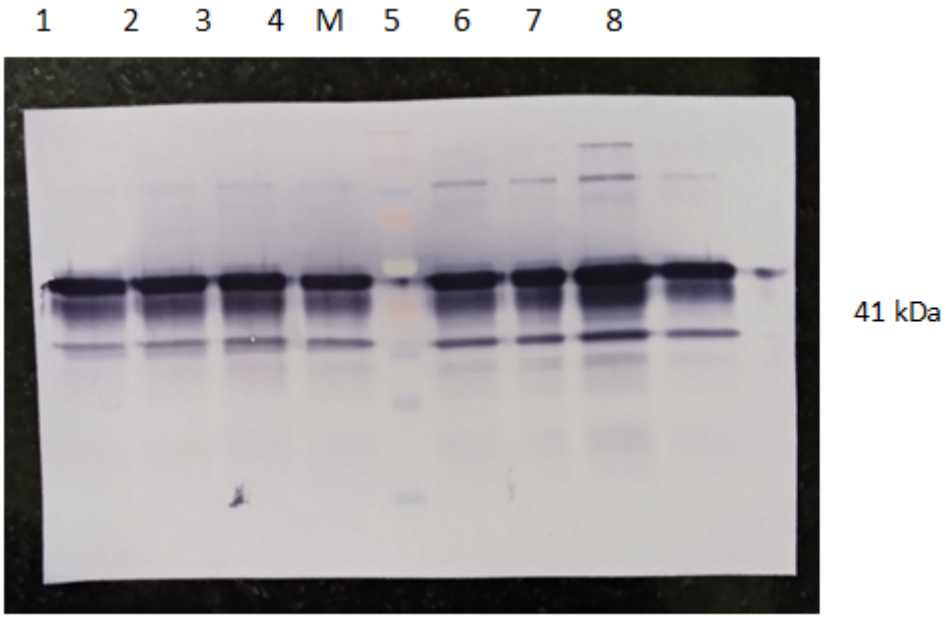

Figure 13

Immuno blot analysis of purified protein extracted from recombinant E. coli BL21 DE3 strain by Western blotting followed by BCIP/NBT staining. M, marker prestained broad range; Lanes 1-4, SPFMV-CP protein eluted using buffer D; lanes 5-8, SPFMV-CP protein eluted using buffer E

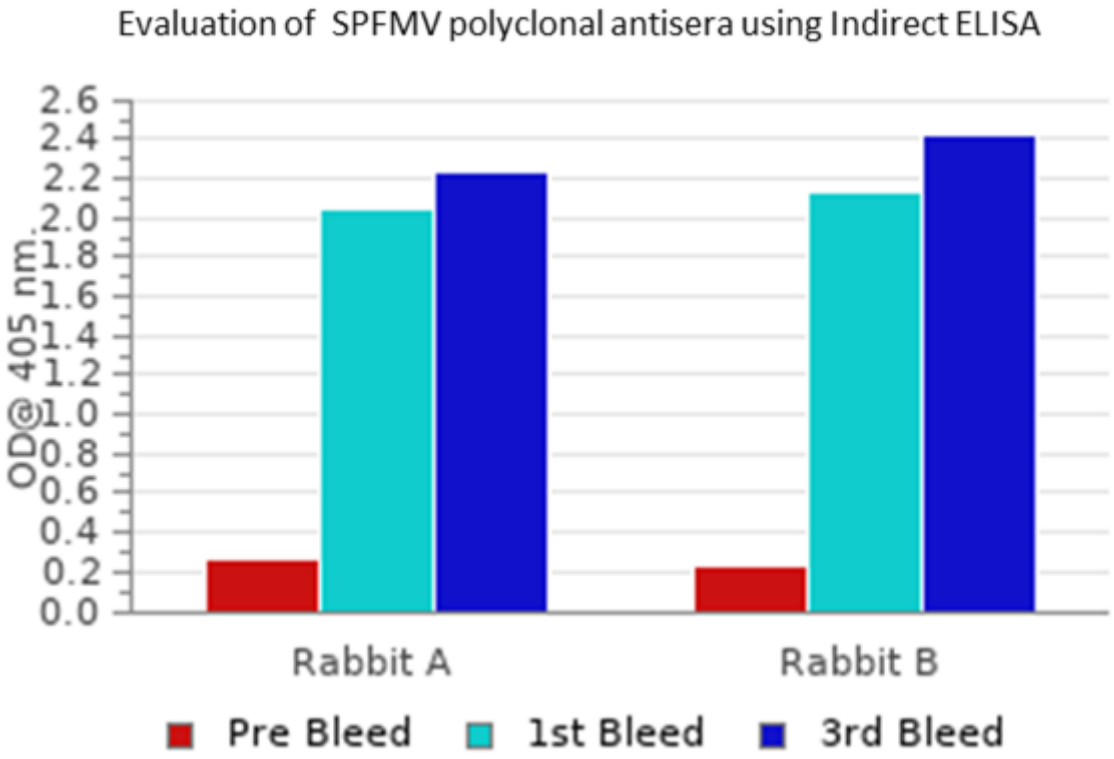

Figure 14

Evaluation of SPFMV polyclonal antisera using Indirect ELISA

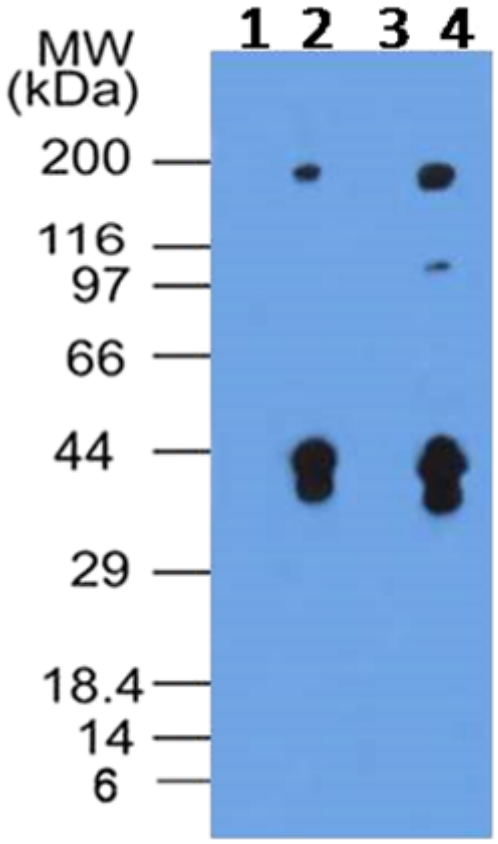

Figure 15

Expression analysis of SPFMV Coat proteinanti-immune sera on custom protein (SPFMV recombinant CP) Lane 1, pre immune sera of Rabbit A tested on supplied protein (20 ng) at 1: 50,000 dilutions; lane2, first immune sera of Rabbit A tested on supplied protein (20 ng) at 1: 50,000 dilutions; Lane 3, pre immune sera of Rabbit B tested on supplied protein (20 ng) at 1: 50,000 dilutions; Lane 4, first immune sera of Rabbit B tested on supplied protein (20 ng) at 1: 50,000 dilutions

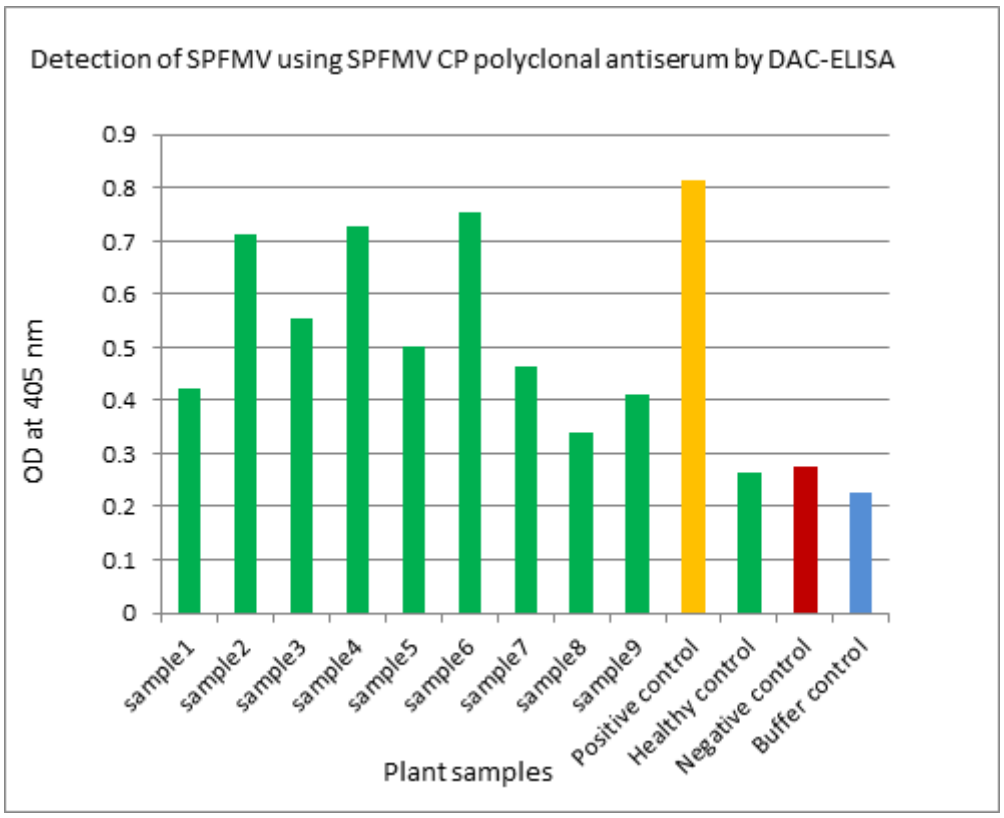

Figure 16

Detection of SPFMV using SPFMV CP polyclonal antiserum by DAC-ELISA

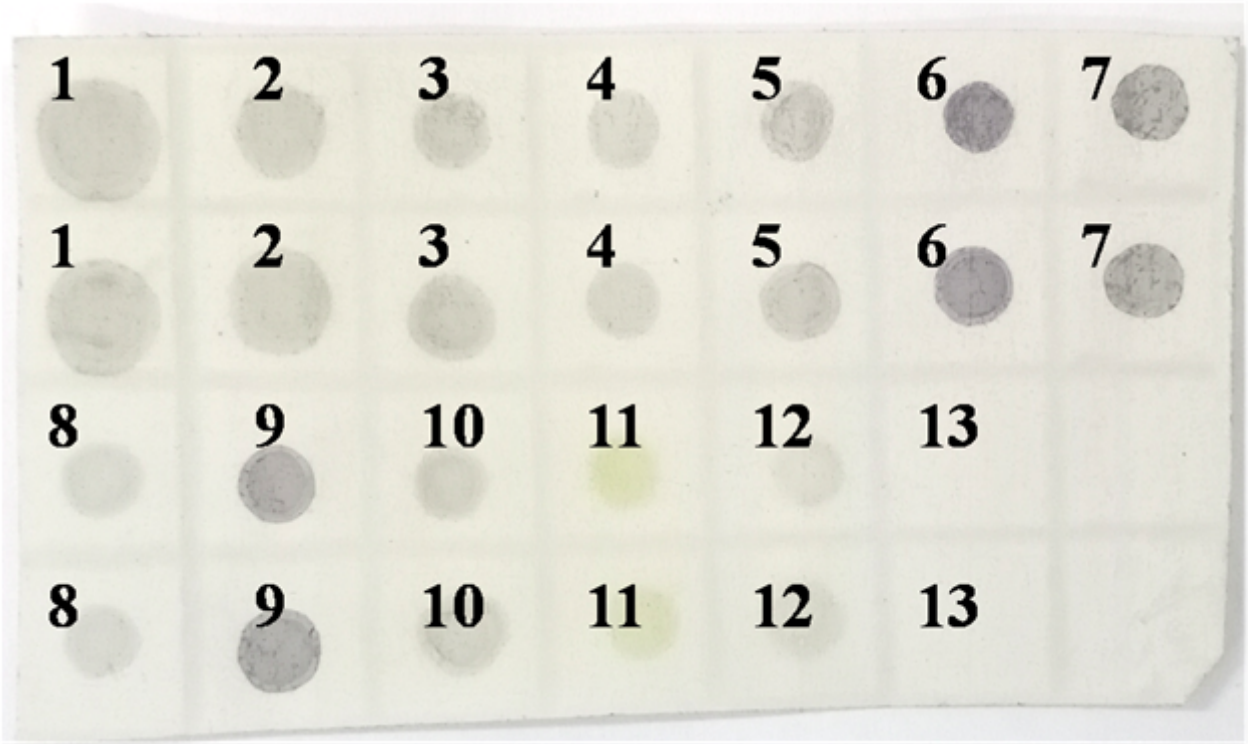

Figure 17

Specificity assay of purified SPFMV polyclonal antiserum using DIBA Lanes 1-9, leaf samples infected with SPFMV; lane 10, positive control; lane 11, healthy plant; lane 12, negative control; lane 13, buffer control

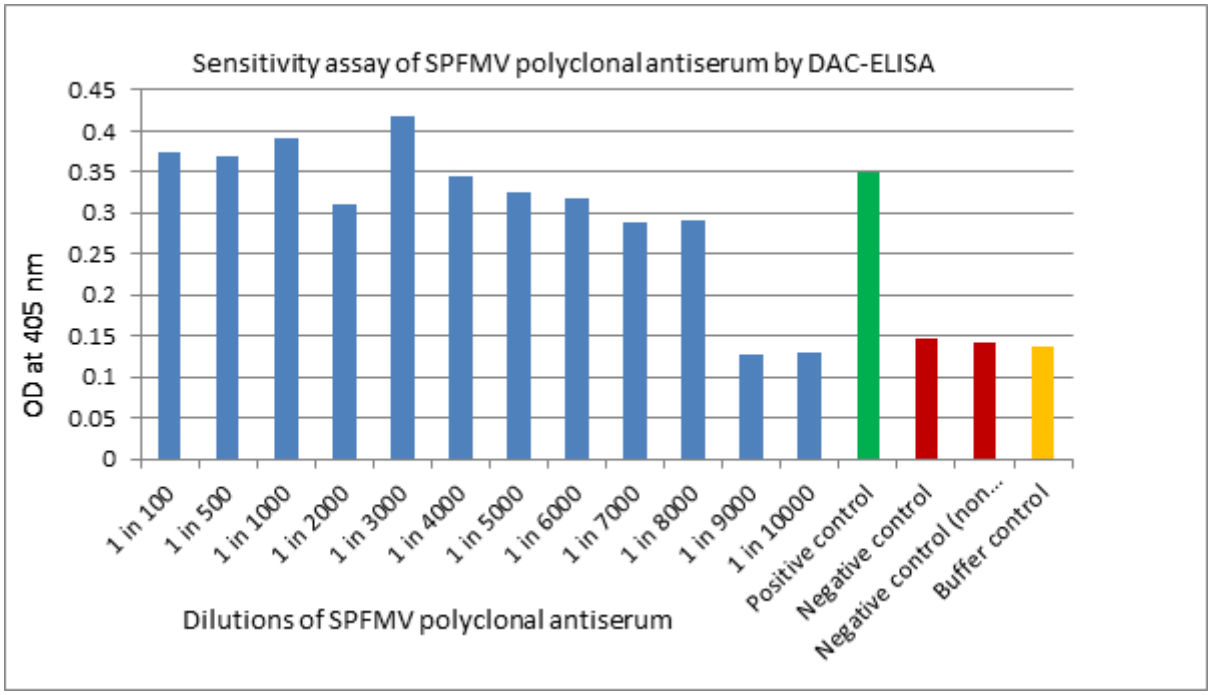

Figure 18

Sensitivity assay of SPFMV polyclonal antiserum by DAC-ELISA