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# Molecular Cloning and Expression of the Coat Protein Gene of *Plum Pox virus* El-Amar Strain in *E.coli*

**M. A. Abou El- Nasr [1]; Kh. A. Dougdoug[1]; Hayam S. Abdelkader[2]; and Rehab A. Dawoud[2]**

*[1]Microbiology Department, Faculty of Agriclture, Ain Shams University, Shoubra El-Khema, Cairo, Egypt.[2]Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza.*

*Plum pox potyvirus* (PPV), the causal agent of Sharka disease of *Prunus*, inflicts severe crop losses in affected El-Amar area. The virus isolated from EL-Amar apricot trees, was propagated on apricot healthy seedlings. Degenerated oligonucleotide primers were designed to amplify the N-terminal portion of the capsid protein of PPV. The amplified products were cloned into pGEM-T-Easy vector and hybridized to PPV DNA specific probe labeled with Dig-11dUTP. DNA sequencing using fluorescent dideoxy nucleotides showed that the capsid protein region of PPV-EA strain had about 65% sequence homology with other strains of PPV and 45% similarity to the CP of PPV-D strain. A PCR fragment coding for the 43 C-terminal amino acids of the Nib and the N-terminal part of the CP (complete variable region plus 19 amino acids of the conserved core) was cloned and expressed into the pQE-100 plasmid vector. Upon induction, the viral protein coat gene was expressed as 6xHis-tagged PPV/CP fusion protein in *E.coli* M15 cells. The fusion protein was confirmed by western blot analysis.

## INTRODUCTION

*Plum Pox virus* (PPV), also known as sharka, is the collective name for a group of devastating viral diseases of stone fruits (Prunus species) including peaches, apricots, plums, nectarines, almonds, and cherries. The disease significantly limits stone fruit production in most areas where it has become established, including large parts of Europe, the Mediterranean, the Middle East (Egypt and Syria), India, and Chile (USDA APHIS PPV Fact sheet, 2001). In North America it was first detected in Pennsylvania in 1998, where it most likely was introduced through infected propagated materials, although the specific pathway and country of origin are unknown (http://sharka.cas.psu.edu). It was detected in Ontario, Canada in 2000, and has not been detected in Mexico. Currently, it has been contained in the U.S. and Canada (Morgan, 2003). Spain was the most recent Western European Country to be invaded by plum pox, in 1984 (Liacer and Cambra, 1998). Although widespread in Europe and USA.*Plum Pox virus* was not reported in Egypt until 1988, when infected trees were observed in plum and peach orchards in El-Amar area (Dunez, 1988).

*Plum Pox virus* is constitutes a virus species in the genus *Potyvirus* in the family *Potyviridae* (Shukla *et al.*, 1994). Rod-shaped PPV particles are about 700 nm long and 15 nm wide. Structurally; potyviruses are about 93 percent proteins and 7 % RNA (Brunt *et al.* 1999). The elongated RNA molecule makes up the genome of the virus and the sequence of nucleotides on the RNA encode 7 different proteins that the virus uses to infect the host, to be transmitted by aphids, and to replicate itself. Once PPV is inoculated into a plant by a vector aphid, the replicating virus can spread

throughout the plant infecting all tissues, including leaves, flower parts, buds, young bark, and roots.

Symptoms induced in stone fruits by PPV may vary considerably with the host plant species, the particular cultivar, age of the plant, nutrient status of the plant, and environmental conditions. In addition, different strains or variants of PPV may vary in virulence, resulting in different degrees of disease severity. Diagnostic symptoms on leaves may consist of mild light green discoloration bordering the leaf veins (vein yellowing) or chlorotic light green or yellowed rings. Fruits of peach and apricot may develop lightly pigmented chlorotic rings or line patterns resulting from several rings coalescing together (Gildow, 2000).

*Plum Pox virus* is known to occur in several different forms or variants called strains. At this time, four distinct major strains have been identified and designated as PPV-D, PPV-M, PPV-C, and PPV-EA. These strains can be distinguished serologically based on differences in the virus coat protein antigenicity and by nucleic acid probes that detect differences in the RNA nucleic acid sequence making up the genome of the different virus strains. The most common European strains reported are PPV-D and PPV-M (Dallot *et al.*, 1998).

The availability of sensitive and efficient detection technique is one of the keys to successful use of virus control strategy. Currently, the virus is detected using a variety of techniques, from visual inspection of fruit growing area to immuno-assays using polyclonal or monoclonal reagents (Cambra *et al.*, 1994) or PCR tests (Wetzel *et al.*, 1991a, 1992; Levy and Hadidi, 1994; Olmos *et al.*, 1996, 1997; and Salama *et al.*, 2003).

In this study, the nucleotide sequence of the coat protein gene of PPV-Amar strain and the use of hybridization and RT-PCR based detection methods for PPV was reported. Moreover, a newly modified PPV/CP constructs were designed according to the conserved amino acid motif DDAGCP using an *E.coli* heterologous expression system. These constructs were expressed in *E.coli* in order to check for the accumulation of PPV/CP. Our results, demonstrated that the strategies developed here provide an effective means of the utility of fusion proteins expressed in *E.coli* as a tool for producing polyclonal antibodies for the diagnosis of *Plum Pox virus* in Egypt.

## MATERIALS AND METHODS

### Source of Virus

The virus was isolated from infected apricot trees growing in El-Amar region, Kalubia Governorate, Egypt and exhibiting typical symptoms of PPV. Field inspections for symptoms observation and the collection of samples were made in April, 2002. These samples were examined serologically by enzyme linked immunosorbent assay (ELISA) (Clark and Adams, 1977) and the positives ones were used for virus propagation by mechanical inoculation of infected sap to healthy *Prunus armeniaca*.

### Amplification of PPV/NIb/CP coding sequence by RT-PCR

Total nucleic acids were isolated from the infected apricot leaves by using a method described by Gibbs and Mackenzie (1997). Sample preparation for amplification of PPV/RNA was performed by grinding 50-100 mg fresh or frozen leaf tissues in liquid $N_2$ to a fine powder and 500 µl of washing buffer (10mM Tris-HCl

pH 8.0, 1mM EDTA pH 8.0, 2M NaCl) was added to the powdered leaves, centrifuged immediately at maximum speed for 10 min. Supernatant was removed and 600µl of CTAB buffer (2 % (w/v) CTAB, 1.4 M NaCl, 0.1 M Tris-HCl pH 8.0) containing 0.5% β-mercaptoethanol was added. The mixture was mixed well and incubated at 55°C for 15-30 min. 400 µl of chloroform: isoamyl alcohol (24:1) was added to the mixture and vortexed to emulsify. The mixture was centrifuged at maximum speed for 10 minutes and the aqueous (top) phase was removed to a clean Eppendorf tube and 1/10$^{th}$ volume of 7.5M $NH_4OAc$ and 1 volume of isopropanol were added. The tubes were mixed well and placed in freezer for 5-10 min. Nucleic acids were pelleted by centrifugation at maximum speed for 5-10 min. Supernatant was poured off and 1 ml of 70% ethanol was added and the tubes were centrifuged for 1min and the pellet was air dried. The pellet was resuspended in 50-100 µl of sterile nuclease free $H_2O$ on ice or overnight at 4°C. The nucleic acids were stored at -20 °C.

cDNA synthesis was carried out in a reaction mixture (25 µl final volume) containing 2 µl of freshly prepared RNA for template; 10 pmol of Oligo (dT) antisense primer; 20 U of M-MuLV reverse transcriptase (Promega); 100 mM of each dNTP; 1 mM DTT; 50 mM Tris–HCl, pH 8.3; 75 mM KCl and 6 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 1 h and stored at -20°C until use. PPV-specific products were amplified by PCR, using UNOII thermal cycler from Biometra, Inc.) in a 25 µl volume containing 5 µl of cDNA; 100 pmol of each primer PV2 and PPVR (Table 1); 100 mM of each dNTP; 1 U of Taq polymerase; 10 mM Tris–HCl, pH 9.0; 50 mM KCl; 1 mM $MgCl_2$ and 0.1% Triton X-100. Denaturation at 93°C for 3 min was followed by 35 cycles of 30 s at 93°C; 30 s at 55°C and 1 min at 72°C, with a final extension step at 72°C for 5 min. Conditions for the amplification of hemi-nested products were the same, except that 100 pmol of specific primers (PPVF and PPVR) and 1 µl of the 1000-fold diluted first PCR reaction mixture for template were used.

| Primer Name | Primers sequence 5' 3' → | Nucleotide position | Size of PCR | |
|---|---|---|---|---|
| PV2 | G(T/C/G)AAA(C/T)AA(C/T)AG(C/T)GG(G/T/A)CA(A/G)CC | 4867-4886 | 985 bp | |
| PPVR | CTTGGCTGCAGGTGC TTCAAGCGTGGC ACTG | 3902-3881 | | 472 bp |
| PPVF | TACGGATCCTACATTGCAGAGACAGCA CTG | 3429-3450 | | |

**Cloning of PPV- Nib/CP into pGEM-T-Easy vector**

The Nib/CP PCR products were ligated into a pGEM-T vector (System I) from Promega (Madison, WI, USA), and transformed into competent JM109 *E. coli* cells (Promega) with subsequent ampicillin selection following manufacturer's instructions. The Promega pGEM-T-Easy cloning kit offered a rapid and efficient cloning method for PCR products based on the use of the T overhang in the vector and A tail on the PCR products as an alternative to the DNA overhangs. After bacterial transformation, 100 µl of the transformation mix were plated onto Luria-Bertani (LB) plates containing 100 µg/ml ampicillin, 50µg/ml X-gal and 100 mM IPTG. At least 10 clear ampicillin-resistant white colonies were randomly picked from each plate and separately cultured for 16 hours at 37 °C in 3 ml of Luria-

Bertani (LB) medium containing 100 µg/ml ampicillin. The small cultures were grown overnight at 37 °C. Plasmid DNA was prepared from each culture by using the Wizard Miniprep Kit (Promega, Madison, WI. USA), following manufacturer's instructions.

**Southern blot-hybridization**

Southern blotting technique was performed on the amplified PPV/PCR fragments of partial sequence of CP/Nib genes that resolved on an agarose gel as described by Southern (1975). The gel was soaked first in 0.4 M NaOH to denature the DNA and then placed in neutralization solution (0.5 M Tris-HCl pH7.4, 1.5 M NaCl). The nitrocellulose membrane and a stack of paper towels were placed over the gel overnight. The paper towels soaked up the buffer, carrying the DNA toward the membrane, in a vertical direction. The DNA strands stick to the membrane in exactly the same relative position that had on the gel. DNA was cross linked onto the membrane in a UV-cross linker between 2.500 and 10.000 µJoules/cm$^2$ for 3 min.

**Probe preparation**

The digoxigenin -11- dUTP–labeled cDNA probes, corresponding to PPV-El Amar CP/Nib genes were amplified by using 10X DNA labeling nucleotide mix (Roche, Boehringer Mannheim, Indianapolis) and as described by Crosslin *et al.* (1992). Digogxigenin-11-dUTP nucleotide mix was incorporated into the PCR cocktail instead of the normal nucleotide mix using the protocol described under the technical bulletin (Roche, Boehringer Mannheim, Indianapolis). The PCR reaction was performed in 50 µl total volume containing 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 1 ng of PPV/CP-Nib cDNA, 1X dNTPs labeling mixture (10mM dATP, 10mM dCTP, 10mM dGTP, and 6.5 mM dTTP, 3.5 mM Dig-11dUTP, in Tris-HCl, pH7.5), 1 µM of PPVR reverse primer (5'-CTTGGCTGCAGGTGCTTTCAAGC GTGGCACTG-3') and 1 µM of PV2 forward primer (5'-G(T/C/G)AAA(C/T)AA(C/T)AG(C/T) GG(G/T/A)CA(A/G)CC-3') and 1.25 U Taq DNA polymerase and then the volume was completed to 50 µl with nuclease free water. The PCR reagents were from (Roche Boehringer Mannheim, (Indianapolis). The PCR was performed for 35 cycles of denaturation at 94°C for 30 sec, annealing at 55 °C for 45 sec, and extension at 72 °C for 1 min.

**Dot-blot hybridization**

0.1 gm of PPV infected leaves were homogenized in 12 x SSC (1X SSC= 150mM NaCl, 15 mM Sodium Citrate, pH 7.0) containing 6% formaldehyde as reported by Hadidi *et al.* (1990) prior to blotting on nitrocellulose membrane. The extracted sap was serially diluted in 6X SSC buffer ($10^0$, $10^{-1}$, $10^{-2}$,$10^{-3}$,$10^{-4}$ and $10^{-5}$) heated to 95 °C for 10 min and chilled on ice. 10 µl of each dilution was spotted onto the membrane using the protocol described by Koreschineck *et al.* (1991). The membrane was denatured by using 0.5M NaOH for 5 min followed by neutralization in neutralization buffer (1M Tris-HCl pH 7.4, 2X SSC) and finally precipitated by ethanol 95% for 5 min and air dried. The nucleic acids were cross-linked to the nitrocellulose membrane at 2,500 µ Joules/cm$^2$ for 3 min. The prehybridization, hybridization, and colorimetric detection procedures were carried out according to the manufacturer's instruction (Roche, Boehringer Mannheim, Indianapolis). The membrane was introduced for colour detection in 20 ml of colour solution (1M Tris-HCl, pH 9.5; 5M

NaCl; 1 M $MgCl_2$) using 35 µl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 45 µl of Nitro blue tetrazolium (NBT) to visualize the dot signals. The membrane was incubated for 15 min in a suitable clean box in the dark. The reaction was stopped when desired signals were obtained using tap water. The membrane was air dried and stored at room temperature.

**Automated DNA Sequencing**

Two clear colonies containing the recombinant plasmids with the CP inserts were re-cultured separately in 50 ml LB medium with ampicillin at 100 µg /ml. Plasmids were extracted using Wizard Plasmid Mini-prep Kit (Promega, Madison, WI. USA). The DNA inserts were verified by (1) PCR using M13F/M13R and /or PPVR/PV2 primers to validate cloning; (2) sequencing of one strand on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) located at the Center for Biomolecular Resource Facility (BRF), Department of Molecular Biosciences, John Curtin School of Medical Science, Australian National University (JCSMR) by using ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

**Cloning and Expression of PPV/CP gene**

The CP gene of PPV was amplified by PCR using the two primers PPVF (5'-TACGGATCCTACATTGCAGAGAC AGCACTG-3') and PPVR (5'-CTTGGCTGCAGGTGCTTTCAAGC GTGGCACTG-3'). The amplified fragment 472 bp was purified from agarose gel electrophoresis by using GFX-Gel Extraction Kit (Pharmacia). It was then cut by Pst1/Bam HI restriction endonuclease enzymes and then ligated into Pst1/Bam HI digested pQE-100 expression vector (Qiagen). Recombinant plasmids containing the inserted PCR fragment were selected by restriction analysis and also validated by PCR to confirm the integrity of the cloned DNA. In this way, the viral cDNA sequence was inserted in frame downstream of the 6X His-protein. Fusion proteins were expressed and purified as described in the QIA Expressionist Manual (Qiagen: Whale *et al.*, 1999). Fractions were analyzed by electrophoresis in 12% SDS-polyacrylamide gels as described by Laemmli (1970).

**Western blot analysis of PPV fusion protein**

Induction of the synthesis of the PPV fusion protein was performed as recommended by QIA expressionist manual (Qiagen). Briefly, exponential phase cultures of *E.coli* M15 cells containing the appropriate recombinant clones were induced by addition of 0.3mM IPTG. Following 1 h. 30 min incubation period at 37 °C with vigorous shaking, cells were harvested by centrifugation (3 min at 13.000 rpm, 4 °C). The bacterial cells were directly resuspended in denaturing polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris-Cl pH 6.8; 10% SDS; 25% B-mercaptoethanol) and lysed by boiling for 5 minutes. Protein extracts were finally clarified by a 10 min centrifugation at 13.000 rpm and either used directly or stored at -20 °C until used. Protein samples were separated on 12% SDS- PAGE gels as described by Laemmli (1970) and transferred by semi-dry blotting on nitrocellulose membranes. The membrane was blocked in TBS (20 mM Tris-HCl pH7.4, and 150 mM NaCl) containing 3% BSA for one hour at room temperature. The blot was incubated with the Ni-NTA alkaline phosphatase conjugate diluted (1:5000) in blocking

buffer for one hour at room temperature and washed three times for 15 minutes each in TBS containing 0.05% Tween-20. The colour reaction was started by incubating the membrane in 5-bromo-4-chloro-3-indolyl phosphate/nitro bluetetrazolium (BCIP/NBT) substrate for alkaline phosphatase until the protein bands have reached the desired intensity. The reaction was stopped by washing the membrane in deionized water for several minutes. The membrane was air dried on a filter paper and photographed.

**Purification of 6xHis-tagged PPV fusion proteins**

Purification of 6xHis-tagged PPV fusion protein was performed under denaturing conditions by nickel-nitrilotriacetic acid (Ni-NTA) batch chromatography as described by Whale *et al.* (1999). The cells pellet from 200 ml of IPTG induced bacterial culture was resuspended in 4 ml of lysis buffer (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris-Cl, pH 8.0). The cell lysate was centrifuged for 10 min at 14.000 xg to remove the cellular debris. To the supernatant, 1 ml of 50% Ni-NTA slurry was added and the lysate-resin mixture was loaded into an empty column. Endogenous proteins with histidine residues were washed out of the matrix twice by 4 ml of washing buffer (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris-Cl, pH 6.3). The 6xHis-tagged fusion protein was eluted twice with 0.5 ml of elution buffer (8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris-Cl, pH 4.5). The 6xHis-tagged fusion protein was dissociated from the Ni-NTA resin at the acidic pH.

**RESULTS AND DISCUSSION**

The genome of the *Plum pox Potyvirus* (PPV) consists of ss-RNA 9,786 nucleotides (nt) long with the potential to encode a 355 kDa polyprotein (Lain *et al.* 1988) which, by proteolytic processing, would originate the mature PPV structural and nonstructural proteins. In this study, the PPV was isolated from apricot trees growing under field conditions in El-Amar area. Mechanically inoculated apricot seedlings exhibited systemic symptoms; i.e. chlorotic fleck mosaic, vein banding, yellowish mottling and leafroll; gave positive results against PPV antiserum by DAS-ELISA. The quality of RNA isolated during this study depended on the source of the tissue being used as starting material. The protocol described under the materials (Gibbs and Mackenzie, 1997) was used successfully to isolate a high yield of total RNAs from infected apricot tissues. The RNA was reverse transcribed by the MMLV reverse transcriptase using Oligo (dT) as minus-sense primer and the resulting complementary DNA (cDNA) was amplified by PCR after adding PV2 and PPVR primers to amplify 985 bp fragment (CP/Nib) (Fig. 2) or PPVF and PPVR to amplify a fragment corresponding to the region of the PPV genome covering the last 43 C-terminal amino acids of the Nib and the N-terminal part of the CP (complete variable region plus 19 amino acids of the conserved core (472 bp), these results were in agreement with that obtained by Candress *et al.* (1998). Figure (1) shows the RT-PCR products obtained from total RNAs extracted from apricot trees infected with PPV by using primers PPVR/PV2. The expected product size was about 985 bp.

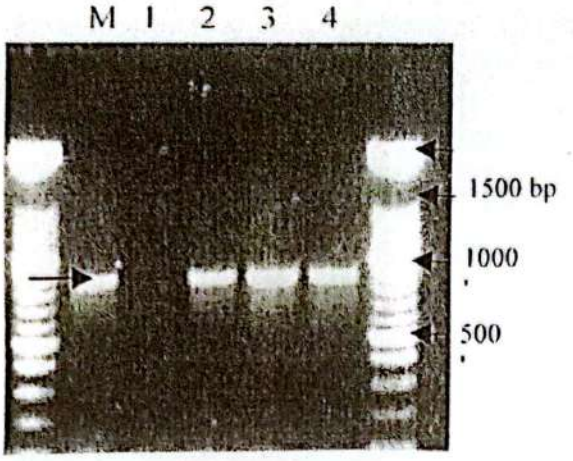



Figure (1): 1% agarose gel electrophoresis showing the RT-PCR products of the CP-Nib region of plum pox virus (PPV) using Hi-expand -Fidelity PCR system. 200 ng of total RNA extracted from infected apricot tissues were reverse transcribed into cDNAs using Oligo (dT) minus primer. Lanes (1, 3, 4 and 5): RT-PCR products of correct size (985 bp) amplified using PPVR and PV2 primers as indicated by arrow. Lane M: Molecular weight Marker (250 bp ladder). Lane 2: Healthy apricot tissues.

Four white colonies resistant to ampicillin containing recombinant plasmids pRPH18, pRPH19, pRPH21 and pRPH32 were selected for testing the presence of PPV/Nib-CP by PCR. PCR products were amplified using both PPVR/PV2 and M13F/M13R primer pairs as shown in Figure (2 and 3). Figure (3 B) shows the Southern hybridization of the amplified products by using specific digoxigenin labeled DNA probe to confirm the authenticity of the PCR products.

Southern blot hybridization technique was used to confirm the authenticity of the PCR products of PPV CP/Nib region obtained. The results verified that DNA probe was successfully hybridized with PPV PCR products, therefore, confirming the authenticity of the amplified PPV/CP coding sequence (Fig. 3 b). The recombinant clones which exhibited the right orientation of the PPV/Nib inserts in PCR amplification using the primer pair M13 forward and M13 reverse (Fig 3) were undergo hemi-nested PCR amplification using the primer pair PPVF and PPVR to amplify the 472 bp PCR products for cloning into pQE-100.

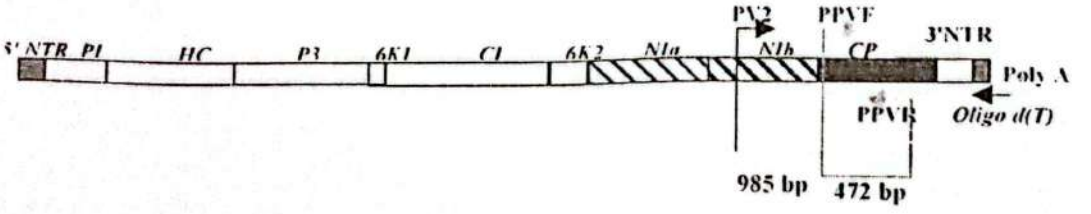



Fig. (2). Cistron map of the potyviral genome. HC, CI, NI, and CP indicate helper component, cylindrical inclusion, nuclear inclusion, and capsid proteins, respectively. Primers used for PCR amplification of partial codon sequences from both the CP and the Nib genes are indicated by arrows. PV2 with PPVR give a PCR fragment size 985 bp and PPVF with PPVR gives a PCR fragment size of 472 bp. Oligo d(T) primer was used in reverse transcription reaction as minus primer.

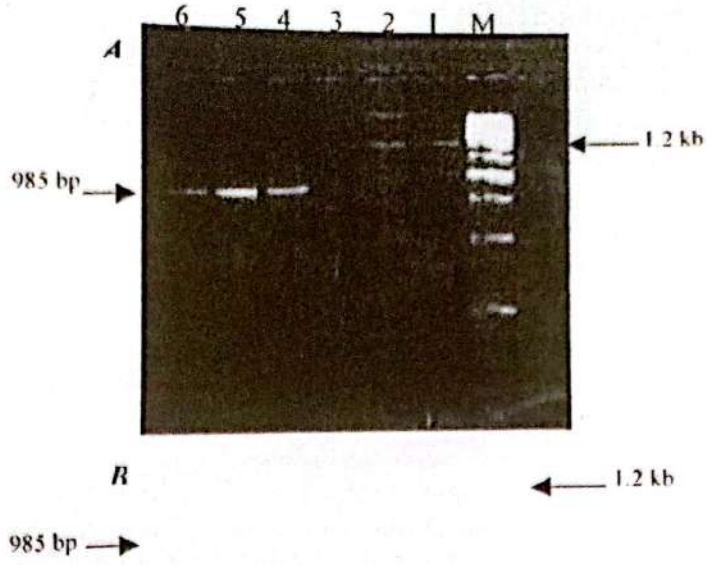
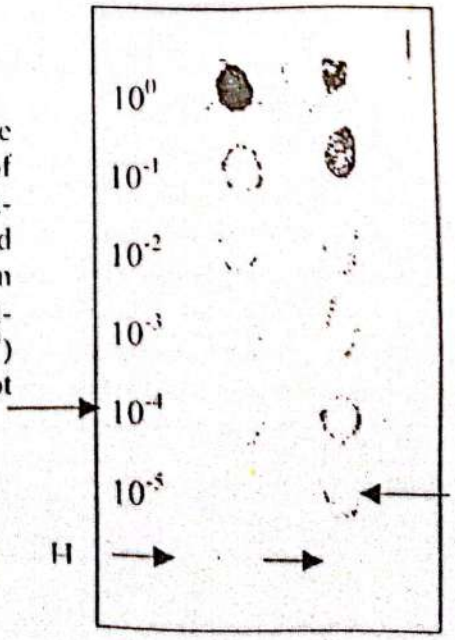


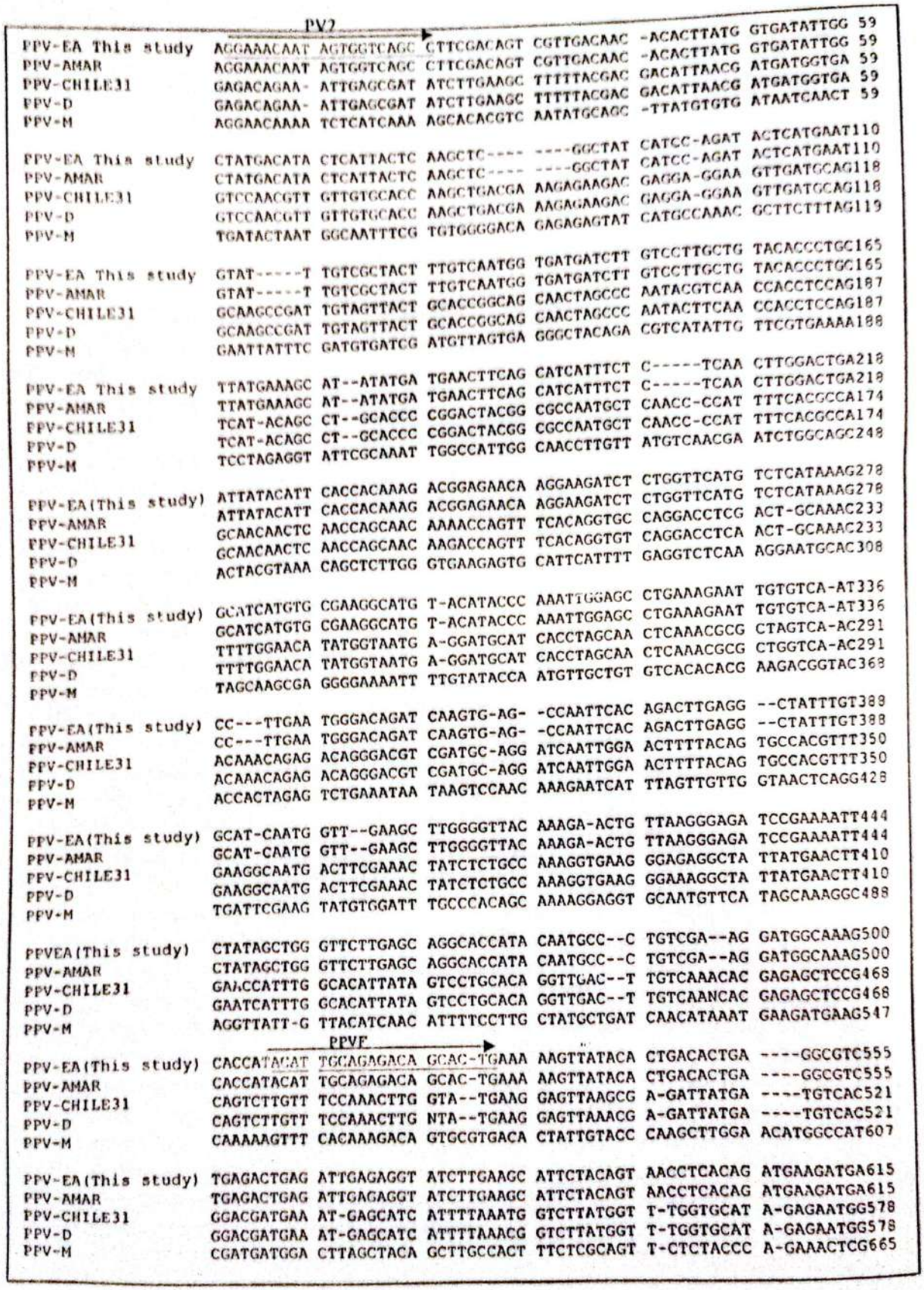

Figure (3): (A) 1% agarose gel electrophoresis showing PPV/CP-Nib PCR products after cloning into pGEM-T-Easy vector using Hi-expand Fidelity PCR system. PCR was performed on recombinant PPV clones to verify the presence of CP/Nib gene insert in pGEM-T plasmid vector. Lanes 1, 2, and 3 showing the expected size (1.2 Kb) of the amplified PCR products using M13 universal primers. Lanes 4, 5, and 6 showing the amplified PCR products of the correct size (985 bp) of PPV/CP-Nib gene insert using the specific primers PPVR and PPV2. (B) Nitrocellulose membrane showing the hybridization signals of both PCRs using specific PPV probe labeled with Digogxigenin - 11-dUTP nucleotide mix. M: Molecular weight DNA Marker (Roche).

Figure (4) Dot-blot hybridization assay showing the colored spots of hybridized nucleic acid extracted from of PPV infected apricot tissues using PPV-CP/Nib DNA-probe labeled with dig-11-dUTP. 10 µl of extracted nucleic acids were spotted in serial dilution on nitrocellulose membrane. The detection limit of dot blot-hybridization assay in row 1 (R1) was at dilution ($10^{-4}$) and $10^{-5}$ dilution in row 2 (R2). H: Healthy apricot tissues showing no signals.



PPV-infected and un-infected apricot leaf extracts were used for dot blot hybridization experiment. 10 µl of leaf extracts (as described under M&M) were spotted onto nitrocellulose membrane at dilutions $10^{0}$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ (Figure 4). The DNA probe enabled detection up to $10^{-5}$ dilutions while no reaction was observed at this dilution with ELISA. On the other hand, the DNA probe gave no signal with extracts of healthy apricot.

Nucleotide sequence analysis (Fig. 5) shows that the nucleotide sequences of the Nib/CP of the virus under study (PPV-EA) and PPV-Amar reported by Wetzel *et al.* (1991 b) were found to be very similar, displaying 100% identity while, displaying 45% similarity with PPV-Chilie31 and PPV-D and 38% identity with PPV-M. The PPV-CP/Nib inserts of three recombinant DNA molecules were chosen for nucleotide sequence analysis. The relationship of PPV-CP/Nib gene sequence Amar strain to PPV-D was shown in Fig. (5 B). About 985 nucleotide of PPV-CP/Nib was cloned and sequenced for comparison with PPV-Amar (Wetzel *et al.*, 1991b), PPV Chilie 31, PPV-D and PPVM using DNAMAN program (Wisconsin, Madison, USA) (Fig. 5 A). The nucleotide sequence of PPV EL-Amar strain (this study) has 100% identity to Amar isolate of Wetzel *et al.* (1991b), 45% with PPV- Chilie 31 and PPV-D and 38 % with PPV-M. A phylogenetic tree of the PPV-EA sequences reveals low degree of similarity to the other three isolates sequences of PPV (Fig. 5b). A prokaryotic system was used to study the impact of the introduced PPV/CP in *E. coli*. Once produced in *E. coli*, the CP was purified using Ni-NTA resin batch chromatography under denatured conditions. The expressed fusion CP was recognized by Ni-NTA alkaline phosphatase conjugate. These observations are in agreement with the model proposed by Shukla *et al.* (1989). When extracts of *E. coli* cells harboring the recombinant plasmid pPVQE-100, which encodes about 472 nt of PPV cDNA, were separated in an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with NiNTA-AP conjugate only a PPV fusion protein band of 37 kDa was detected (Fig. 6A, lanes E1 and E2), indicating that efficient expression and purification was taking place at the right open reading frame. This fusion protein expressed in *E.coli* was similar in size with that obtained by Candress *et al.* (1998). The SDS-PAGE and western blot analysis of the purified fusion protein CP/Nib (Fig. 6 A and B) revealed the presence of a major protein band with an estimated molecular mass of 37 KDa, confirming that CP insert was really fused to the His-tag. The bacterial cells were harvested and the presence of the desired protein was proved by western blotting using Ni-NTA alkaline phosphatase conjugate against 6x-His-tag (Fig.6B). The different aliquots (cleared cell lysate, flow through, and eluted 6x-His-CP fusion protein) collected during Ni-NTA batch protein purification were subjected to SDS-PAGE and western blot analysis as presented in Fig. (6A and B).


PV2
PPV-EA This study AGGAAACAAT AGTGGTCAGC CTTCGACAGT CGTTGACAAC -ACACTTATG GTGATATTGG 59
PPV-AMAR AGGAAACAAT AGTGGTCAGC CTTCGACAGT CGTTGACAAC -ACACTTATG GTGATATTGG 59
PPV-CHILE31 GAGACAGAA- ATTGAGCGAT ATCTTGAAGC TTTTTACGAC GACATTAACG ATGATGGTGA 59
PPV-D GAGACAGAA- ATTGAGCGAT ATCTTGAAGC TTTTTACGAC GACATTAACG ATGATGGTGA 59
PPV-M AGGAACAAAA TCTCATCAAA AGCACACGTC AATATGCAGC -TTATGTGTG ATAATCAACT 59
PPV-EA This study CTATGACATA CTCATTACTC AAGCTC---- ----GGCTAT CATCC-AGAT ACTCATGAAT110
PPV-AMAR CTATGACATA CTCATTACTC AAGCTC---- ----GGCTAT CATCC-AGAT ACTCATGAAT110
PPV-CHILE31 GTCCAACGTT GTTGTGCACC AAGCTGACGA AAGAGAAGAC GAGGA-GGAA GTTGATGCAG118
PPV-D GTCCAACGTT GTTGTGCACC AAGCTGACGA AAGAGAAGAC GAGGA-GGAA GTTGATGCAG118
PPV-M TGATACTAAT GGCAATTTCG TGTGGGGACA GAGAGAGTAT CATGCCAAAC GCTTCTTTAG119
PPV-EA This study GTAT-----T TGTCGCTACT TTGTCAATGG TGATGATCTT GTCCTTGCTG TACACCCTGC165
PPV-AMAR GTAT-----T TGTCGCTACT TTGTCAATGG TGATGATCTT GTCCTTGCTG TACACCCTGC165
PPV-CHILE31 GCAAGCCGAT TGTAGTTACT GCACCGGCAG CAACTAGCCC AATACGTCAA CCACCTCCAG187
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PPV-M GAATTATTTC GATGTGATCG ATGTTAGTGA GGGCTACAGA CGTCATATTG TTCGTGAAAA188
PPV-EA This study TTATGAAAGC AT--ATATGA TGAACTTCAG CATCATTTCT C-----TCAA CTTGGACTGA218
PPV-AMAR TTATGAAAGC AT--ATATGA TGAACTTCAG CATCATTTCT C-----TCAA CTTGGACTGA218
PPV-CHILE31 TCAT-ACAGC CT--GCACCC CGGACTACGG CGCCAATGCT CAACC-CCAT TTTCACGCCA174
PPV-D TCAT-ACAGC CT--GCACCC CGGACTACGG CGCCAATGCT CAACC-CCAT TTTCACGCCA174
PPV-M TCCTAGAGGT ATTCGCAAAT TGGCCATTGG CAACCTTGTT ATGTCAACGA ATCTGGCAGC248
PPV-EA(This study) ATTATACATT CACCACAAAG ACGGAGAACA AGGAAGATCT CTGGTTCATG TCTCATAAAG278
PPV-AMAR ATTATACATT CACCACAAAG ACGGAGAACA AGGAAGATCT CTGGTTCATG TCTCATAAAG278
PPV-CHILE31 GCAACAACTC AACCAGCAAC AAAACCAGTT TCACAGGTGC CAGGACCTCG ACT-GCAAAC233
PPV-D GCAACAACTC AACCAGCAAC AAGACCAGTT TCACAGGTGT CAGGACCTCA ACT-GCAAAC233
PPV-M ACTACGTAAA CAGCTCTTGG GTGAAGAGTG CATTCATTTT GAGGTCTCAA AGGAATGCAC308
PPV-EA(This study) GCATCATGTG CGAAGGCATG T-ACATACCC AAATTGGAGC CTGAAAGAAT TGTGTCA-AT336
PPV-AMAR GCATCATGTG CGAAGGCATG T-ACATACCC AAATTGGAGC CTGAAAGAAT TGTGTCA-AT336
PPV-CHILE31 TTTTGGAACA TATGGTAATG A-GGATGCAT CACCTAGCAA CTCAAACGCG CTAGTCA-AC291
PPV-D TTTTGGAACA TATGGTAATG A-GGATGCAT CACCTAGCAA CTCAAACGCG CTGGTCA-AC291
PPV-M TAGCAAGCGA GGGGAAAATT TTGTATACCA ATGTTGCTGT GTCACACACG AAGACGGTAC368
PPV-EA(This study) CC---TTGAA TGGGACAGAT CAAGTG-AG- -CCAATTCAC AGACTTGAGG --CTATTTGT388
PPV-AMAR CC---TTGAA TGGGACAGAT CAAGTG-AG- -CCAATTCAC AGACTTGAGG --CTATTTGT388
PPV-CHILE31 ACAAACAGAG ACAGGGACGT CGATGC-AGG ATCAATTGGA ACTTTTACAG TGCCACGTTT350
PPV-D ACAAACAGAG ACAGGGACGT CGATGC-AGG ATCAATTGGA ACTTTTACAG TGCCACGTTT350
PPV-M ACCACTAGAG TCTGAAATAA TAAGTCCAAC AAAGAATCAT TTAGTTGTTG GTAACTCAGG428
PPV-EA(This study) GCAT-CAATG GTT--GAAGC TTGGGGTTAC AAAGA-ACTG TTAAGGGAGA TCCGAAAATT444
PPV-AMAR GCAT-CAATG GTT--GAAGC TTGGGGTTAC AAAGA-ACTG TTAAGGGAGA TCCGAAAATT444
PPV-CHILE31 GAAGGCAATG ACTTCGAAAC TATCTCTGCC AAAGGTGAAG GGAGAGGCTA TTATGAACTT410
PPV-D GAAGGCAATG ACTTCGAAAC TATCTCTGCC AAAGGTGAAG GGAAAGGCTA TTATGAACTT410
PPV-M TGATTCGAAG TATGTGGATT TGCCCACAGC AAAAGGAGGT GCAATGTTCA TAGCAAAGGC488
PPVEA(This study) CTATAGCTGG GTTCTTGAGC AGGCACCATA CAATGCC--C TGTCGA--AG GATGGCAAAG500
PPV-AMAR CTATAGCTGG GTTCTTGAGC AGGCACCATA CAATGCC--C TGTCGA--AG GATGGCAAAG500
PPV-CHILE31 GAACCATTTG GCACATTATA GTCCTGCACA GGTTGAC--T TGTCAAACAC GAGAGCTCCG468
PPV-D GAATCATTTG GCACATTATA GTCCTGCACA GGTTGAC--T TGTCAANCAC GAGAGCTCCG468
PPV-M AGGTTATT-G TTACATCAAC ATTTTCCTTG CTATGCTGAT CAACATAAAT GAAGATGAAG547
PPVF
PPV-EA(This study) CACCATACAT TGCAGAGACA GCAC-TGAAA AAGTTATACA CTGACACTGA ----GGCGTC555
PPV-AMAR CACCATACAT TGCAGAGACA GCAC-TGAAA AAGTTATACA CTGACACTGA ----GGCGTC555
PPV-CHILE31 CAGTCTTGTT TCCAAACTTG GTA--TGAAG GAGTTAAGCG A-GATTATGA ----TGTCAC521
PPV-D CAGTCTTGTT TCCAAACTTG NTA--TGAAG GAGTTAAACG A-GATTATGA ----TGTCAC521
PPV-M CAAAAAGTTT CACAAAGACA GTGCGTGACA CTATTGTACC CAAGCTTGGA ACATGGCCAT607
PPV-EA(This study) TGAGACTGAG ATTGAGAGGT ATCTTGAAGC ATTCTACAGT AACCTCACAG ATGAAGATGA615
PPV-AMAR TGAGACTGAG ATTGAGAGGT ATCTTGAAGC ATTCTACAGT AACCTCACAG ATGAAGATGA615
PPV-CHILE31 GGACGATGAA AT-GAGCATC ATTTTAAATG GTCTTATGGT T-TGGTGCAT A-GAGAATGG578
PPV-D GGACGATGAA AT-GAGCATC ATTTTAAACG GTCTTATGGT T-TGGTGCAT A-GAGAATGG578
PPV-M CGATGATGGA CTTAGCTACA GCTTGCCACT TTCTCGCAGT T-CTCTACCC A-GAAACTCG665

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PPV-EA(This study) ATCCAATGTA GTTGTCCATC AAGCTGATGA GAAGGAAGAC GATGAAGAGG AAGTGGA---672
PPV_AMAR           ATCCAATGTA GTTGTCCATC AAGCTGATGA GAAGGAAGAC GATGAAGAGG AAGTGGA---672
PPV-CHILE31        AACATCCCCG AATATCAATG GAATGTGGGT GATGATGGAT GGGGAAACAC AAGTGGAGTA638
PPV-D              AACATCCCCG AATATCAATG GAATGTGGGT GATGATGGAT GGGGAAACAC AAGTGGAGTA638
PPV-M              GAATGCTGAG CTTCCACGAA TACTCGTTGA TCATGAAGCA AAGATCTTTC ATGTGGTTGA725

PPV-EA This study) TGCAGGGAGG CCTTTAGTCA CCACTACACA GCAGCCAATT GTTACCACAA CAACTCAGCA732
PPV-AMAR           TGCAGGGAGG CCTTTAGTCA CCACTACACA GCAGCCAATT GTTACCACAA CAACTCAGCA732
PPV-CHILE31        TCCAATAAAG CCATTGTTGG --ATCATGCG AAACCCACTT TTAGACAAAT TA---TGGCA693
PPV-D              TCCAATAAAG CCATTGTTGG --ATCATGCG AAACCCACTT TTAGACAAAT TA---TGGCA693
PPV-M              CTCATTCGGA TCACTGTCAA CTGGAATGCA TGTTTTGAAA GCGAACACAA TCAATCAACT785

PPV-EA(This study) AACT------ -CCAATAACG -----AGTAC AACCTT---- ACAAGCTACG CAGGCAATGT776
PPV-AMAR           AACT------ -CCAATAACG -----AGTAC AACCTT---- ACAAGCTACG CAGGCAATGT776
PPV-CHILE31        CATT------ -TCAGTAACG T----GGCTG AAGCGT---- ATATTGAAAA ACGAAATTAT738
PPV-D              CATT------ -TCAGTAACG T----GGCTG AAGCGT---- ATATTGAAAA ACGAAATTAT738
PPV-M              TATTAGCTTT GCTAGTGATA CATTGGATTC AAGCATGAAA ACATACCTGG TTGGAGGTCT845

PPV-EA(This study) TTAATCCCAT CTTCACTCCA GCGACGACTG AGCCGACCAC TAGGACAGTG CCTCACACAA836
PPV-AMAR           TTAATCCCAT CTTCACTCCA GCGACGACTG AGCCGACCAC TAGGACAGTG CCTCACACAA836
PPV-CHILE31        GAAAAAGCAT ACATGCCAAG GTATGGAATT CAGCGCAACC TGACAGACTA CAGC-CTCGC797
PPV-D              GAAAAAGCAT ACATGCCAAG GTATGGAATT CAGCGCAACC TGACAGACTA CAGC-CTCGC797
PPV-M              TGAAGTGGAT AAGTGTGATG AATTCAAAAA TGTCAAGCTC TTGATCAGAA GCATTTACAA914

PPV-EA(This study) ----CAACTA CTACACCTCC TTCTTTTGGA GTGA---TCG GGAATGAGGA TACCG-CACC888
PPV-AMAR           ----CAACTA CTACACCTCC TTCTTTTGGA GTGA---TCG GGAATGAGGA TACCG-CACC888
PPV-CHILE31        ----CAGATA -TGCCTTTGA TTTTTACGAA ATGACT-TCA ACGACACCCG TACGGGCACG851
PPV-D              ----CAGATA -TGCCTTTGA TTTTTACGAA ATGACT-TCA ACGACACCCG TACGGGCACG851
PPV-M              GCCACAAATC ATGGAGCAGG TGCTTAAGGA AGAACCATAT TTACTGCTCA ATAGTGGTTC974

PPV-EA This study) CAATGCTTCC AATGCAGTAG TTCGAACAG- GTAGAGACAG AGATGTTGAT GCGGGTTCCA947
PPV-AMAR           CAATGCTTCC AATGCAGTAG TTCGAACAG- GTAGAGACAG AGATGTTGAT GCGGGTTCCA947
PPV-CHILE31        TGAAGCTCAT ATCCAGATGA AGGCAGCAGC ATTGAGAAGT GTTCAAAATC GTTTATTTGG911
PPV-D              TGAAGCTCAT ATCCAGATGA AGGCAGCAGC ATTGAGAAAT GTTCAAAATC GTTTATTTGG911
PPV-M              TGAGCGTTTT GTCACCTGGC GTCTTAATGG CGCTGTTCA- ATTGGAAAAA GCCACACAAT1033
                                                  PPVR
PPV-EA This study) TTGGGACTTT CACAGTGCCA CGCTTGAAAG CA 979
PPV-AMAR           TTGGGACTTT CACAGTGCCA CGCTTGAAAG CA 979
PPV-CHILE31        CTTGGATGGA AACGTCGGAA CACAAGAAGA GG 943
PPV-D              CTTGGATGGA AACGTCGGAA CACAAGAAGA GG 943
PPV-M              ATTGGATTAC ACGATCTCAT AGCTTGGCAG CG1065
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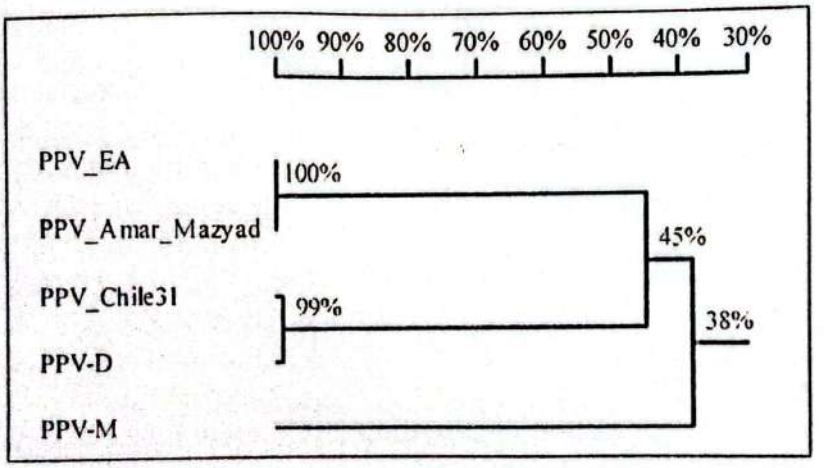


Fig. (5 A &B). Nucleotide sequence alignment of five PPV sequences; PPV-EA (this study), PPV-El-Amar strain (Wetzel *et al.;* 1991b), PPV- Chilie 31, PPV-D and PPV-M using DNAMAN program (Wisconsin, Madison, USA). The nucleotide sequences of the Nib/CP of PPV-EA (this study) and PPV-Amar were found to be very similar, displaying 100% identity. PPV-EA sharing 45% similarity with PPV-Chilie31 and PPV-D and 38% similarity with PPV-M.

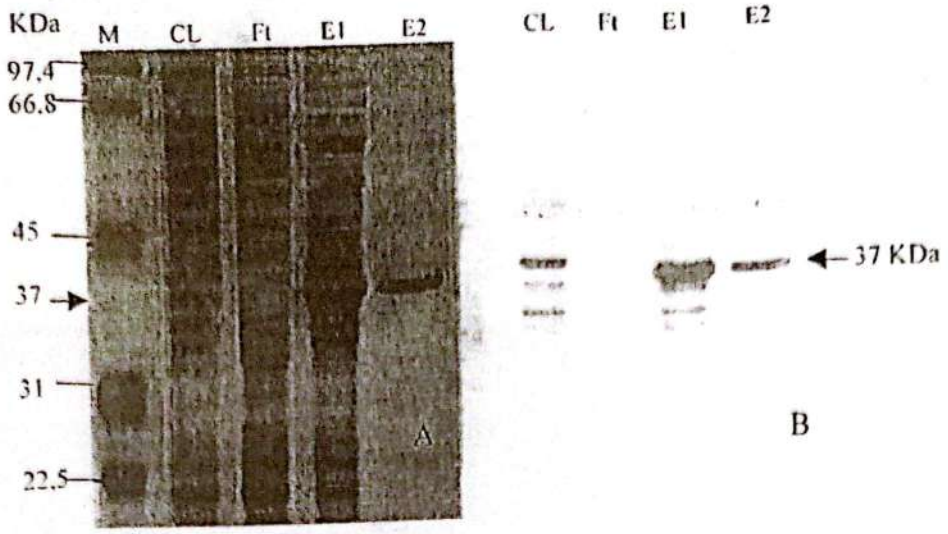


Fig. (6): Cultures of *E.coli* transformed with pQE-100-PPV/CP were induced with 1 mM IPTG, and fusion proteins were all expressed to high levels in soluble form in *E.coli* and analysed by 12 % SDS-PAGE (A) followed by western blotting (B). The results showed that the Ni-NTA alkaline phosphatase conjugate recognized bands of the expected size 37 kDa (6xHis=0.84 KDa; PPV/CP=36 KDa). M: Protein Molecular weight Marker, CL: Cleared lysate of induced culture, Ft: Flow through, E1 & E2: First and second elution of purified 6xHis/CP fusion protein.

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