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Cloning, Expression and Nucleotide Sequence of Coat Protein Gene of an Egyptian Isolate of *Potato Virus Y* Strain NTN Infecting Potato Plants

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Reverse transcription- polymerase chain reaction (RT-PCR) amplified product of the expected size of 801 bp was cloned into the Pin Point Xa-1 protein expression vector. The accury of each PCR amplified PVY CP gene was tested by PCR, restriction analysis and translation. Analysis by *in vitro* translation using western blotting assay on nitrocellulose membrane using monoclonal antibodies verified that the PVY-CP gene correctly encoded and expressed a protein reacting with PVY antibodies. The CP gene approximately 32 KDa reacted successfully with PVY specific monoclonal antibodies in dot blot immunobinding assay (DIBA) as well as in western blot assay. The nucleotide sequence of the Egyption isolate of PVY CP gene was determined to be 801 nucleotides in length encoding a deduced 267 amino acid. Nucleotide sequence analysis revealed a range of 96- 99.5 % sequence identity among the PVY ^{NTN} isolate (PVY–T, PVY SWS, PVY H and PVY ^{NTN}).

INTRODUCTON

Potato Virus Y (PVY) is the type membre of the potyvirus group, the largest group of RNA plant viruses (Holling & Brunt, 1981 and Matthews, 1985). PVY is consisted of a complex group of isolates and strains that are distinguishable by host range, symptomatology and serology. Strains have been identified that cause significant if not devasting diseases of potato and tobacco (Vorster *et al.*, 1990 ; Heath *et al.*, 1987 ; Latorre *et al.*, 1982; DeBokx &Huttinga, 1981 ; Gooding & Lapp , 1980 and Silberschmidt , 1960). On the basis of local and systemic symptoms in different potato cultivars and indicator plant species the PVY isolates have classically been divided into three main strains (DeBokx & Huttinga , 1981). PVY^O (common strain), PVY^N (tobacco veinal necrosis strain) and PVY^C (stripple streak strain). In Egypt, PVY was isolated from potato plants by several workers from potato plants (Kishtah, 1970; El-Banna, 1985; Shalaby, 1993; Gamal El-Din *et al.* 1997 and Amer, 1999). Several workers isolated PVY all over the world. It was isolated from potato (Calvert *et al.*, 1980; Kurppa, 1983 and Deng *et al.*, 1990). However, some authors have reported the existence of strains that do not fit in this grouping (DeBoks *et al.*, 1975). In the last 25 years, other new strains of PVY have been reported from Europe (DeBokx , 1972 and Chrzanowska, 1994), though PVY ^{NTN} received more attention. This virus was first recorded in Hungary (Beczner *et al.*, 1984) causing a new disease called potato tuber necrotic ringspot disease (PTNRD) that rapidly spread to several countries (Weidemann & Mais 1996 and Gamal El-Din *et al.* 1997). PVY ^{NTN} is considered a subgroup of the PVY N strain group which appears to be different to other isolates reported that produces vein necrosis on tobacco but that does not react with monoclonal antibodies specific to PVY N. The PVY genome is composed of a single –stranded positive sence RNA approximately 10 kilo bases (Kb) in length, with a genome link protein (VPg) at the 5- terminus, and a poly A single at the 3- terminus (Dougherty & Hiebert, 1980).

The present investigation aimed to describe the cloning and sequencing of the coat protein coading region of the local isolate of PVY ^{NIN}, and its expression in *E. coli*. A comparison study was carried out between the present isolate and other recorded ones in GenBank.

MATERIALS AND METHODS

Virus isolate Source, maintenance and RNA extraction

Potato (*Solanum tuberosum* L. cvs. Nicola and Spunta) plants showing a mild mosaic were collected from Kafr El-Zayayt, Gharbia Governorate, then tested by direct double antibody sandwich – enzyme linked immunosorbent assay (DAS-ELISA) against PVY as demonstrated by Clark &Adams (1977). ELISA kit's was supplied by SANOFI. Sante Animales. France. Infected plants were maintained in tobacco (*Nicotiana tabacum* L.cv. White Burley) plants by mechanical sap inoculation within an aphid-proof cage under normal glasshouse conditions. Three weeks after inoculation, leaves were harvested and total RNA was extracted from infected and uninfected plant materials using SV – total RNA isolation system (Promega Corporation, Madison, WI).

Reverse transcription–polymerase chain reaction (RT-PCR) amplification of the PVY-CP gene

RT-PCR was done with Access RT- PCR system (Promega Corp, Madison, USA) according to the manufactory instruction's. The oligonucleotide primer used designed according to Sudarsono *et al.* (1993). The complemantary primer (5`- gga tcc aca tgt t(c/g) act cca ag ( t/c) ag-`3) and the homologous primer (5`- gga tcc atg g (g/c) aaa tga cac aat (t/c) gat gca –`3) .The thermal cycling profile 30 cycles of denaturation at 94 °C for 1 min followed by annealing at 52 °C for 1 min . and primer extension at 72 °C for 2 min , with a final extension step of 72 °C for 7 min. PCR amplification was performed in an Thermoblock cycler PCR . (Biometra).

For electrophoretic analysis, aliquots 10 ul each of PCR amplified DNA products were mixed withe gel loading buffer. Separation was done on a 1% agarose gel in 1XTBE buffer (1x = 89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.3). DNA was stained with ethidium bromide added to the gel at a concentration of 0.5 ug / ml. DNA was visualized on a UV transilluminator and photographed using DNA documentation gel analysis. DNA marker (50 bp) (Promega) was used to determine the size of RT- PCR amplified cDNA products.

Cloning and transformation into protein expression vector

PVY cDNA amplified product was cloned using the Pin Point Xa-1 vector cloning system (Promega Corp, Madison . USA) . *E. coli* strain JM 109 was transformed with the recombinant plasmid. DNA was prepared from selected white colonies were screened by PCR and digestion analysis with Bam HI then fractionated on agarose gels.

Protein expression.

Expression protein was visualized using media containing Isopropyl–β-D-Thiogalactopyranoside (IPTG). Total proteins were analyszed by denaturing sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) . The protein gel was stained and visualized by silver nitrate staining (Sambrook *et al.*,1989). Polypeptides resolved on an SDS-PAGE were transferred onto a nitrocellulose membrane using a transblot apparatus (Bio-Rad) as described by Towbin *et al.* (1979) . According to Swank & Munker (1971), the membrane was transferred into blocking solution for 1 h at 37°C. The

blocking solution was replaced with TBST containing the producing antibody and incubated at 37 °C for 2 h . The membrane was washed 5 times (each for 5 min) with TBS-Tween-20 (20 mM Tris- HCl , pH 7.4 , 150 mM NaCl containing 1 % Bovn serum albumin and 0.05 %Tween-20) to remove the unbounded antibody. TBST was added containing the conjugated antibody (ELISA kit's was supplied by SANOFI, Sante Animales, France) to the membrane and incubated at 37 °C for 2 h, then washed 5 times (each for 5 min) with TBST and the membrane was rinsed briefly with water. The color reaction was started by incubating the membrane in freshly prepared NBT (Nitro blue tetrazolume) / BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate for alkaline phosphatase at room temperature with gentle agitation until purple bands appear.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.

Sequencing and computer analysis

The nucleotide sequence of CP-PVY clone was carried out in Biotechnology Department , Molecular Plant Virology Group,The Royel University for Agricultural and Veternr , Denmark) and determined according to the method of (Beek *et al.*, 1993). Coat protein gene sequences from the following virus stored isolates from GeneBank (National Center for Biotechnology Information (NCBI), National Institute of Health (NIH) were used for comparison: [PVY Chilean (X68221), PVY US (X68222), PVY Europe H (X68223) , PVY^{O} (X68226) (Sudarsono *et al.* 1993)] , [PVY Sw (X97895) (Jakab *et al.* 1997)],PVY NTN (AF321554) (Unpublished), PVY^{O} (AF345650) (Unpublished), [PVY^{N} Egypt (AF522296) ((Unpublished)],[PVY French (D 0441)(Robaglia *et al.*1989)], PVY-T(D12570)(Unpublished), [PVY Hungarian (M95491) (Thole *et al.*, 1993)], [PeMV (NC_001517) (Vance *et al.*, 1992)][ZYMV (L31350) (Wisler *et al.*, 1995)] , [TEV (M11458) (Allison *et al.*, 1985)], [PsbMV (NC_001671)(Johansen *et al.*1991)],and [PVV (AJ253119).(Unpublished)] .The sequence data, multiple alignment , and phylogenetic relationship were translated and analyzed by DNA Star Laser gene (DNA STAR Inc. MD) and DNA MAN.

RESULTS AND DISCUSSION

Amplification of the PVY coat protein gene

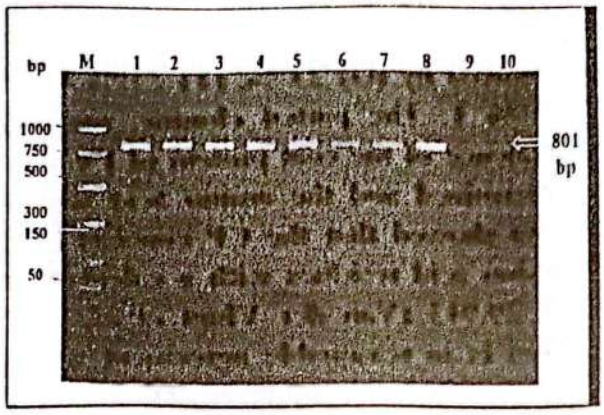
The RT-PCR provides a sensitive and specific technique for rapid detection and amplification of PVY coat protein gene using a specific primer for coat protein, RT-PCR, has been utilized successfully to detect PVY in infected plant materials. The PCR conditions described in this investigation successfully amplified CP gene of the Egyption isolate of PVY^{NTN} Egypt isolate. RT-PCR amplification of viral RNA was carried out on the total RNA isolated from infected and uninfected plant materials using specific primer for PVY –CP designed to amplify 801 bp of the coat protein gene. Electrophoresis analysis of RT-PCR product showed a single amplified fragment of 801 bp (Fig .1), illustrates agarose gel electrophoresis analysis of RT-PCR amplified PVY-CP cDNA from PVY uninfected potato leaves cv. Nicola (lane 1), infected potato leaves and tuber cv. Nicola (lane 2,3), Lane (4) infected *Nicotiana tabacum* cv. White Burly, uninfected potato leaf cv. Nicola (lane 5), infected potato leaves and tuber cv. Spounta (lane 6,7) uninfected tobacco leaves (Lane 6)and uninfected tobacco leaf (lane 8). The RT-PCR method has been also successfully utilized for the detection of PVY in potato tubers (Barker *et al.*, 1987). The RT-PCR or PCR method has been successfully utilized to detect viruses from infected potato tubers (Hadidi *et al.*,

Amplified PVY cDNA –cp was cloned into Pin Point Xa1cloning vector after using Taq DNA polymerase for amplification, whereas the 3` A – overhangs were not removed. The recombinant plasmid was successfully introduced into *E. coli* (JM109 strain) recombinant colonies which had correct insert (801 bp) was validated by: PCR screening conducted on the presumptive recombinant plasmid using the outer plus and minus primer for coat protein (Fig 2). Plasmid minipreparation followed by digestion of plasmid by Bam HI restriction enzymes. Sex colonies were identified and shown to contain insert with the expected molecular weight size (Fig. 3).

The PVY CP gene cloned into the expression vector was expressed in *E. coli*. The expression of CP gene was induced by adding 100 mM IPTG. JM109 *E.coli* strain carring the lac mutation was used for expression of CP-PVY gene. Screening of small scale (3 ml) expression cultures was performed under denaturing conditions in order to identify the ~ 32 KD polypeptide band representing PVY CP gene. SDS – PAGE analysis revelead the presence of a prominent band with an estimated molecular mass of approximately 45 KD (including the 13 KD from plasmid expression blus~32 Kd from protein expressed PVY CP gene) in the total proteins isolated from induced cultures. (Fig 4 A) and the presumptive coat protein gene specifically reacted with monoclonal antibody for PVY N in western blot analysis. (Fig 4 B).

Dot – Immunobinding assay (DIBA)

The induced and noninduced protein of *E. coli* lysate from PVY –CP gene expression were dot blotted onto nitrocellulose membrane. The membrane was soaked in TBS (20 mM Tris base, 500 mM NaCl, pH 7.5) containing 1% bovine serum albumin (BSA) overnight at room temperature and reacted with PVY specific anti rabbit antibody conjugated with alkaline phosphatase (SANOFI, Sante Animales, France). The membrane was subjected to extensive washing in TBS buffer containing 0.1 % Tween-20 and the reaction visualized by incubation in color solution (NBT/BCIB) as substrate (Lizarrage and Fernandez-Northcote, 1989).



Fig(2) Screening of plasmid Pin Point Xa-1 expression vector transformed in *E.coli* .colonies by plasmid minipreparation using PCR. Electrophoresis was performed on agarose gel staining with ethedium bromide. The insert DNA contains the PVY-CP gene fragment from white colonies. (Lanes 1,2,3,4,5,6) The arrow indicated a 801 bp . Lane M, PCR DNA marker (50, 150, 300, 500, 750, 1nd 1000 bp (Promega).No fragment with unligated plasmid (lane 7).

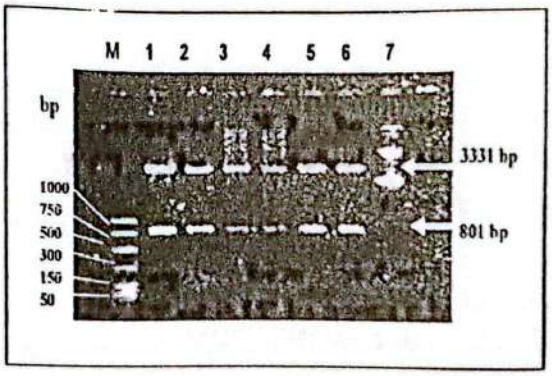


Fig (3) Screening of of plasmid Pin Point Xa-1 expression vector transformed in E.coli .colonies by plasmid minipreparation using restriction digestion enzymes by Bam III. Electrophoresis was performed on agarose gel staining with ethedium bromide The high arrow indicated a ~3.3Kb (plasmid -without insert) , the bottom arrow indicates a 801 bp (PVY CP gene) (lanes 1,2,3,4,5,6,7,8) . No fragment with uncut plasmid (lane 9,10), Lane M, PCR DNA marker (50, 150, 300, 500, 750, 1nd 1000 bp (Promega)

Sequencing of PVY CP gene

The CP coding sequence of the Egyption isolate of PVY ^{NTN} was predicted to be 801 nucleotides in length, encoding a protein of 267 amino acids with a calculated Mr 29.980 Dalton. Fig (5) shown the CP nucleotide sequence and predicted amino acid. The nucleotide composition of the cDNA CP sequence revealed the highest contents for (A) 35 %, followed by (G) 23 %, (T) 23 % and (C) 19 % T. The partial cloning, nucleotide sequence of the coat protein of PVY^{N} was determined and the amino acid sequence data showed that the CP consists of 267 amino acid residues with a calculated Mr of 29.913 (Van der Vlugt *et al.* ,1988). This is in reasonable agreement with the observed Mr 33.000 to 34.000 for PVY as estimated from SDS-PAGE (Huttinga & Mosch, 1974). Rosner & Racch (1988) demonstrated the nucleotide sequence restriction analysis and localization of the CP –PVY gene. The CP region has 801 nucleotide long ending with TGA. This region was located by comparing the predicted amino acid sequence with the one determined for the PVY-CP by (Shukla *et al.*, 1986). Both sequences contained 267 amino acids sharing about 94% homology.

Similarity of CP sequence among different isolates of PVY and potyviruses

Nucleic acid sequence analysis revealed a range of 94.6 to 99.5% sequence identity among the PVY ^{NTN} isolate and other PVY isolates. The highest sequence similarity was found between the isolate under study and PVY-T isolate (99.5 %), while the lowest was found between this isolate and PVY ^{O} isolate (84.6%). The similarity CP sequence was found between the isolate under study and other potyviruses were to be (59.7 to 64 %). The highest sequence similarity was found between this isolate and PeMV (64%) but the lowest was found between this isolate and ZYMV (59.7%). The coat protein of PVY ^{NTN} Egypt isolate is 99.5 % identical to that PVY –T, 98.4 % identical to that PVY SWS, 96.5 % identical to that PVY H, 96.1 % identical to that PVY ^{NTN}, 96.1 % identical to that PVY Europe, 89.3 % identical to that PVY^{O}, 88.8 % identical to that PVY ^{N} Egypt isolate, and PVY Chilean isolate, 88.6 % identical to that PVY Fr, 88 3% identical to that PVY US, 84.6 % identical to that PVY ^{O}, 64 % identical to that PeMV, 63.8 % identical to that PPV, 63 % identical to that PsbMV , 62.1 % identical to that TEV , and 59.8 % identical to that ZYMV. Table (2) shown the percentage sequences similarities between the PVY ^{NTN} CP Egyptian isolate sequence and 11 different PVY isolates and 5 different potyviruses. Shukla & Ward (1988 &1989) proposed the use of CP amino acid sequence as the basis for potyvirus classification . Similarities of 90% to 99% identify the individual viruses as strains, while similarities of 50 to 70 % identify individuals as a distinct virus.

Phylogenetic tree illustrating phylogenetic relationships based on multiple alignment of CP nucleotide sequences of 11 PVY isolates and 5 potyviruses. (Fig 6). Van der Vlugt *et al.* (1993) described the comparison and phylogenetic relationships derived from multiple sequence alignment. Evidence is presented that it is indeed possible to distinguish different strains of one *Potyvirus* species on the basis of both CP cistron and 3'-NTR sequences.

In this report we describe the molecular cloning and nucleotide sequqcing of the CP gene of PVY ^{NTN} (Eg) In addition, we succssed in expressing PVY CP gene derived proteins in *E. coli*. The size of PVY CP gene was 801 nucleotides.

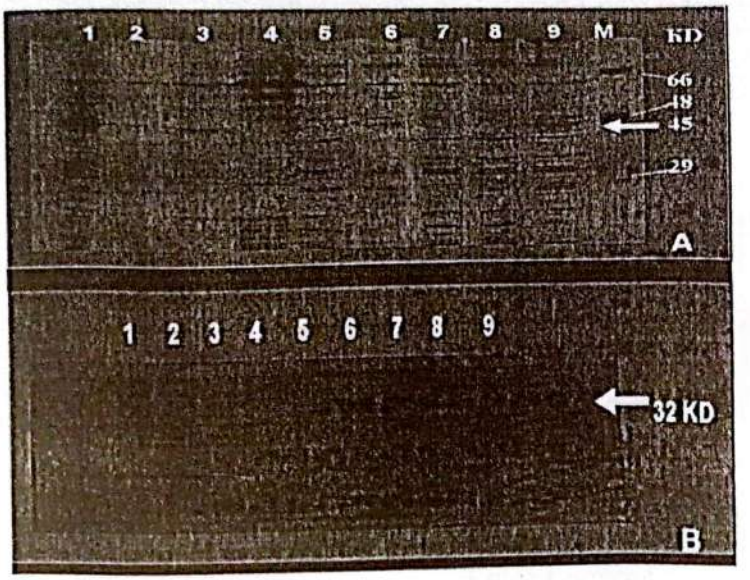


Fig (4) SDS PAGE 12 % analysis showing rapid screening of small (3 ml) expression culture under denaturing conditions and induced by adding 100 mM IPTG. A) a silver nitrate stained SDS-PAGE the total cell proteins with amajor 43 KD polypeptide bads (lanes :1,2,3,4,5,6,7,8,9) . B) Western blot analysis showing a prominent band with an estimated molecular mass of approximately 30 KD representing exprassion coat protein for PVY which specifically reacted with anti mouse . (lane 1,2,3,4,5,6,7,8,9). Lanes 9 , total cell protein showing no PVY-CP expression.

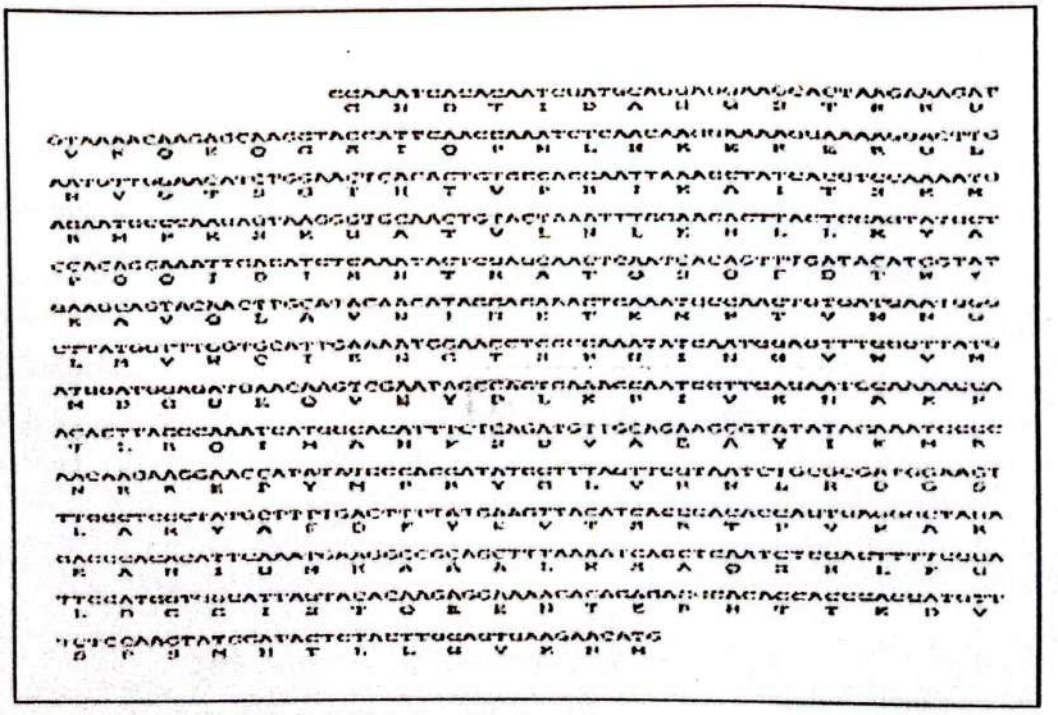
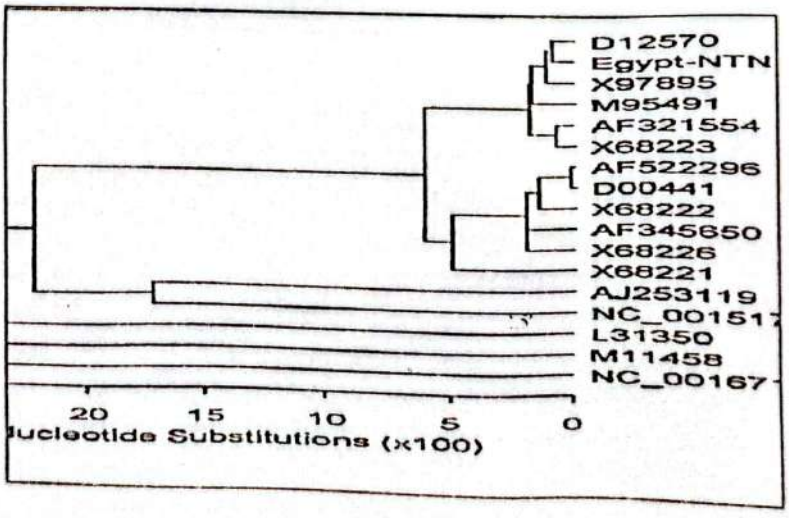


Fig (5) Nucleotide sequence and derived amino acid sequences of the PVY CP Egypt isolate representing the 801 nucleotides.

Table (1): Percentage sequence similarity between the all PVY isolate and potyviruses

EGYPT NTN	100%
X97895	98.4 % 100%
AF321554	96.1% 97.0% 100%
AF345650	84.6% 85.0% 86.2% 100%
AF522296	88.8% 93.0% 90.0% 93.0% 100%
AJ253119	63.3% 63.0% 62.8% 61.2% 62.7% 100%
D00441	88.6% 98.4% 90.7% 92.9% 99.9% 62.5% 100
D12570	99.5% 98.6% 96.4% 86.4% 89.0% 62.3% 88.9% 100%
L31350	59.7% 60.2% 59.6% 57.0% 59.9% 62.6% 59.9% 57.2% 100%
M11458	60.0% 59.9% 60.1% 59.2% 60.7% 57.1% 60.6% 59.8% 61.3% 100%
M95491	96.5% 97.4% 98.9% 86.0% 90.8% 62.8% 90.6% 96.7% 58.9% 60.7% 100%
NC_001517	63.8% 63.5% 63.5% 60.7% 63.2% 64.7% 63.0% 62.5% 62.4% 58.3% 62.3% 100%
NC_001671	63.0% 58.4% 57.6% 55.4% 62.4% 57.2% 62.5% 57.5% 52.1% 58.4% 57.4% 56.7% 100%
X68221	88.8% 89.7% 90.6% 86.1% 91.8% 61.7% 91.6% 89.1% 59.0% 60.2% 90.5% 61.8% 56.7% 100%
X68222	88.3% 88.5% 90.4% 92.4% 97.4% 63.3% 97.3% 86.6% 63.6% 60.3% 90.3% 63.6% 62.2% 90.5% 100%
X68223	96.1% 97.0% 98.7% 85.9% 90.6% 62.8% 90.5% 96.4% 59.1% 60.2% 98.6% 62.9% 57.6% 90.4% 90.2% 100%
X68226	89.3% 89.3% 90.4% 97.6% 62.8% 97.5% 89.3% 59.2% 60.3% 90.8% 69.8% 69.3% 60.3% 91.2% 96.9% 90.9% 100%



(Fig 8) Phylogenetic tree between PVY NTN Egypt isolate and different PVY isolates and potyviruses.

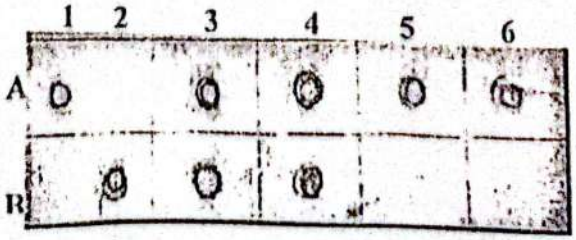


Fig (9): NCM- ELISA of representing expression coat protein for PVY that specifically reacted with goat anti rabbit IgG. Lane A : 1,,3,4,5 and 6 . Lane B : 2,3 and 4), total cell protein showing no PVY –CP expression (lane A : 2) and Lane B : 1,5 and 6).

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