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Original article

Polyclonal antibodies against the recombinantly expressed coat protein of the *Citrus psorosis virus*

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ABSTRACT

Psorosis is a damaging disease of citrus that is widespread in many parts of the world. Citrus psorosis virus (CPsV), the type species of the genus Ophiovirus, is the putative causal agent of psorosis. Detection of CPsV by laboratory methods, serology in particular is a primary requirement for large-scale surveys but their production has been impaired by the difficulty of obtaining sufficient clean antigen for immunization. Specific PAbs against coat protein were produced in *E. coli* using recombinant DNA approach. The full length CP gene fragment was amplified by RT-PCR using total RNA extracted from CPsV infected citrus leaves and CP specific primers. The obtained product (1320bp) was cloned, sequenced and sub-cloned into $p^{ET\text{-}30(+)}$ expression vector. Expression was induced and screened in different bacterial clones by the presence of the expressed protein (48kDa) and optimized in one clone. Expressed CP was purified using batch chromatography under denaturing conditions. Specificity of expressed protein was demonstrated by ELISA before used as antigen for raising PAbs in mice. Specificity of the raised PAbs to CPsV was verified by ELISA and western blotting. The raised PAbs were showed highly effectiveness in screening by ELISA comparing with the commercial antibodies purchased from Agritest, Valanzano, Italy.

The expression of CPsV CP gene in *E. coli*, production of PAbs using recombinant protein as an antigen, the suitability of these antibodies for use in immunodiagnostics against the CPsV Egyptian isolate have been accomplished in this work.

1. Introduction

Citrus is a very important crop in many tropical and subtropical countries throughout the world, however certain virus and virus-like diseases together with fungal and bacterial diseases can limit production and in some cases are destroying and ravaging citrus as an industry or eliminating backyard trees (Martin et al., 2002). Psorosis is a damaging disease of citrus that is widespread in many parts of the world and is the first of the citrus virus diseases described and the oldest researched citrus virus disease (Garcia et al., 1997). Disease development is slow; it may take several years to manifest symptoms, which include bark scaling of the trunk, and chlorotic flecks and spots on young leaves. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion; these symptoms have been used for field diagnosis of Psorosis (Sertkaya, 2014). Citrus psorosis virus (CPsV), the type species of the genus Ophiovirus, is the putative causal agent of psorosis (Garcia et al., 1997). CPsV is a multi-components ssRNA virus with a coat protein of approximately 48 kDa (Barthe et al.,1998; Petrzik et al., 2001; Martín et al., 2006). CPsV genome consists of three ssRNAs of negative polarity, RNA1 contains 8184nt and its complementary strand has two ORFs potentially encoding a 24 kDa protein of unknown function and a 280 kDa protein with motifs characteristic of RNA-dependent RNA polymerases (RdRp). RNA2 contains

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1644nt and its complementary strand encodes a 54 kDa protein (Martín et al., 2005), and RNA3 contains 1454nt and its complementary strand encodes the CP (Garcia et al., 1997; Barthe et al., 1998). For many years, laborious and costly indexing on citrus indicators was the only available diagnostic method (Alioto et al., 1999). Nowadays, ELISA and RT-PCR are the most reliable and rapid methods for detecting the presence of the virus in presumed infected-trees in the field (Martin et al., 2002). The RNA3 of CPsV which encodes for CP of 48KDa protein has been used for detection of the virus (Garcia et al., 1997). Detection of CPsV by laboratory methods serology in particular, is a primary requirement for large-scale surveys such as those carried out for sanitary selection in the framework of certification programs (Onghia et al., 2001). Virus particles are normally used as an antigen for polyclonal and monoclonal antibody production to be used subsequently for serological diagnosis (Elgaied et al., 2017). Their production has been impaired by the difficulty of obtaining sufficient clean antigen for immunization, due to the erratic transmission and low concentration of the virus in herbaceous hosts (Martin et al., 2002). In this paper, the expression of CPsV-CP gene of Egyptian viral isolate in *E. coli*, production of polyclonal antibodies using recombinant protein as an antigen, and the suitability of these antibodies for use in ELISA and western blotting immunodiagnostics have been reported.

2. Materials and methods

2.1. Virus source and CP gene isolation

Twenty-seven (27) samples showing psorosis like symptoms (mottling, ringspot, chlorosis, vein enation, crinkly), bark (bark scaling, gummies, concavities) and fruit symptoms (acorn shaped and ringspot), were collected from citrus trees (cv. navel orange) grown in Qanater area, Qaluobia governorate, Egypt and subjected for detection against CPsV antibodies by DAS-ELISA as described by Clark and Adams, 1977 using monoclonal antibodies (MAbs) specific to CPsV, purchased from Agritest, Valanzano, Italy.

Total RNA was extracted from 50 mg leave tissues of CPsV-infected citrus plants according to the procedure described in tripure isolation reagent manual, Roche, IN, USA. Coat protein gene specific primers were designed according to CPsV sequences available in the genbank database using lasergene (megAlign version 4.0) and primer select version 4.0; CPf GGCG*GGATCC*TCGATTCCTATTAAAGTGT and CPr CGA*AAGCTT*TTACATAGTCGCAGCCA, the consensus nucleotides are underlined, while the italic ones refer to *Bam* H1 and *Hind* III restriction sites in CPf and CPr, respectively.

RT-PCR was carried out using one-step RT-PCR protocol (Qiagen), 2 μg of total extracted RNA, 10 μl of 5× buffer, 2 μl of dNTP mix (containing 10 mM of each dNTP), 2 μl of enzyme mix, and 0.6 μM of each primer in total reaction volume 50 μl adjusted by RNase free dH_2O. RT-PCR cycling was performed in the following conditions: 50°C for 30 min, 94 °C for 15 min, 30 cycles 94 °C, 55 °C and 72 °C for 45 sec, and a final extension at 72 °C for 10 min. Amplified PCR products were analyzed on a 1.4% agarose gel.

2.2. Cloning, sequencing and expression the coat protein gene

The purified RT-PCR product was ligated into the pGEM®-T easy vector system I according to manufacturer's instructions and transformed *E. coli* (JM 109) cells. Recombinant plasmid was miniprepared from overnight grown *E. coli* in LB containing 100 μg/ml Amp. Recombinant and non-recombinant colonies were screened by blue-white selection. Plasmid recombination was confirmed by restriction endonuclease enzymes analysis. Isolated DNA was subjected for cycle sequencing using the fluorescent dideoxy chain terminator technology, big-dye terminator kit and an applied biosystem 373A sequencer. The determined nucleotide sequence was compared with other sequences deposited in the genbank.

The full length CP gene was sub-cloned into the expression vector $p^{ET\text{-}30(+)}$ (Novagen) in N-terminal tag 6xHis using *Bam* H1 and *Hind* III restriction enzymes and transformed into *E. coli* BL21 competent cells.

Expression of the coat protein gene was induced using 1mM IPTG. The presence of expressed CPsV-CP was verified in four randomly selected clones through rapid screening by SDS-PAGE analysis. Expression level was optimized *via* time course, by harvested cells in different times post induction with IPTG and analyzed by SDS-PAGE 12%.

2.3. Purification, specificity assaying and mice injection of the expressed protein

Purification of the expressed coat protein was carried out using Ni-NTA batch chromatography under denaturing conditions. Bacterial cells grown in 200 ml LB media were harvested after 16 h post induction with IPTG and gently resuspended in 4 ml lysis buffer (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl, pH 8.0) to recovering the insoluble fusion proteins under denaturing conditions. Cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The supernatant was passed through Ni-NTA resin column (Qiagen) after incubation with 50% Ni-NTA slurry by gently shaking for 50 min at room temperature. 6xHis proteins were bound to the column in high pH of 8 M urea. The matrix washed out twice and 6xHis proteins were eluted against a pH gradient down to pH4.5, dialyzed against several changes of phosphate-buffered saline (PBS) solution at 4 °C and analyzed by SDS-PAGE. The concentration of purified protein was estimated using Bradford method. Specificity of the purified expressed proteins was assayed as antigen by DAS-ELISA against CPsV specific MAbs purchased from Agritest, Valanzano, Italy.

Antiserum was raised against expressed 6Xhis-tagged CPsV-CP by immunize Balb-C mice upon the approval of AGERI internal biosafety and bioethics committee. Six weeks old mice were administered by 100 μg expressed coat protein in a primary response; followed by four weekly intraperitoneal injections of 200 μg without remove of 6xhis-tag, where cleavage of the N-terminal fusion protein was not necessary as this not expected to have significant immunogenic properties (Mutasa-Gottgens et al., 2000). Mice were emulsified with an equal volume of freund's complete adjuvant for the first injection and incomplete adjuvents for the four subsequent intramuscular injections at weekly intervals. The blood was collected after 5 injections and incubated at 37 °C for 1h then centrifuged at 4000 rpm.

2.4. Evaluation the raised antibodies comparing with the commercial

Specificity of the raised antiserum was evaluated as trapped antibody by ELISA in a dilution 1:5,000 against bacterial cell extracts from transformed bacteria with recombinant and non-recombinant plasmids, total extracted proteins from healthy and CPsV-infected citrus plants as well as the purified fusion proteins. Similarly, western blotting was used to evaluate the raised antiserum against the same mentioned antigens. 12% SDS-PAGE was prepared and the polypeptides were transferred onto a membrane (immobilon® PVDF membrane, millipore cooperation, Bed ford, MA 01730) using a trans-blot apparatus (Bio-Rad). The membrane was blocked in TBS containing 5% BSA, and then the blocking buffer was replaced with the TBS containing raised antibodies (1:5,000). After incubation, the membrane was washed with TBST, and incubated in TBS containing anti-mouse universal antibodies and detection was developed by NBT/BCIP in alkaline phosphatase buffer.

Table 1
The detection of CPsV infected citrus plants (27 samples) by ELISA, using the commercial purchased polyclonal antibodies:

Sample no.	ELISA reading values	Sample no.	ELISA reading values	Sample no.	ELISA reading values
1	0.300	10	0.298	19	0.393
2	0.222	11	0.256	20	0.403
3	0.254	12	0.223	21	0.305
4	0.345	13	0.277	22	0.265
5	0.242	14	0.256	23	0.273
6	0.298	15	0.387	24	0.399
7	0.298	16	0.344	25	0.740
8	0.207	17	0.430	26	0.391
9	0.621	18	0.367	27	0.305
P. control	0.692	N. control	0.280		

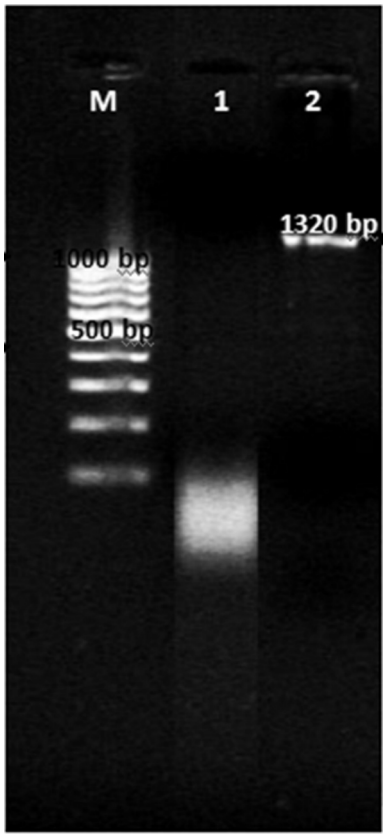

Fig. 1. PCR amplification for CPsV CP gene. M: 100bp ladder. 1: healthy citrus tissues. 2: infected citrus tissues.

The 27 citrus samples which previously screened by ELISA using the commercial antibodies purchased from Agritest, valanzano, Italy, were screened twice again using our raised antiserum as trapped and intermediate antibody *via* plate-trapped antigen indirect (PTA-I) and double antibody sandwich (DAS) ELISA respectively.

3. Results

3.1. Virus detection and cloning the CP gene

Two out of the 27 collected samples were reacted positively in ELISA, against specific CPsV commercial antibodies upon OD_{405} readings comparing with the negative control (healthy citrus plant) (Table 1). Citrus samples reacted positively in ELISA referred to citrus trees exhibited bark scaling and gum symptoms. Total RNA was extracted from sample No. 25 (Table 1), plus healthy citrus tissues as a negative control. The full length of CP gene fragment (1320bp) was amplified from infected citrus plants RNA extracted and no amplification from healthy citrus plants RNA extracted (Fig. 1).

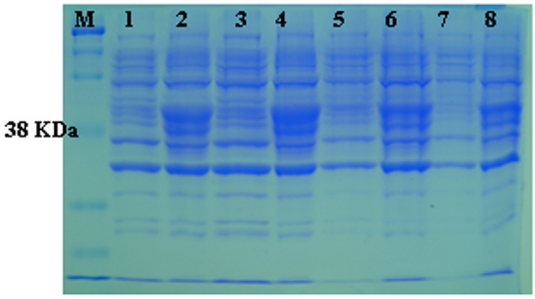

Fig. 2. Screening the presence of the expressed CPsV-CP in extracted bacterial cell lysates in four clones (selected randomly) transformed with recombinant plasmid M: pre- stained protein marker. 1, 3, 5 and 7 represents the four clones before induction; 2, 4, 6 and 8 the same clones after induction.

Sequence of the isolated DNA fragment was determined and submitted to GenBank under accession number KM206785. Alignment of isolated sequence revealed 98% level of similarity with other CPsV CP gene sequences.

3.2. Expression of CPsV CP gene

The bacterial cells harboring the generated expression plasmid with the CPsV-CP gene after induction with IPTG, were analysed by SDS-PAGE. One major band with apparent molecular weight 48 kDa, revealed the main difference in SDS-PAGE analysis between the bacterial cell lysates before and after induction with IPTG. This band was clearly appeared after induction in all of selected clones expressing the coat protein gene (Fig. 2).

Expression Level of CPsV-CP was optimized via time course analysis for the expressed protein by SDS-PAGE in different times (1, 2, 3, 4, 5 and 16 h) post induction with IPTG, showed increasing in intensity of the expected band (48 kDa) to reach high accumulation after 16 h post induction with IPTG (Fig. 3). To this end, expression experiments confirmed the presence of expressed CPsV-CP fused with 6xHis in N-terminal, as expected using translation option of Lasergene software (data not shown).

3.3. Purification and assaying the expressed coat protein

The coat protein fused with 6xHis in N-terminal was purified from the bacterial cell lysate by affinity chromatography using His-Select Nickel Affinity Gel. The purified coat protein produced one distinct Pure band with approximately molecular weight 48 kDa (Fig. 4). Protein concentration was estimated by a total amount 3 mg expressed CP purified from 200 ml culture by Bradford assay and spectrophotometry. The purified CP (1.5 mg/ml)

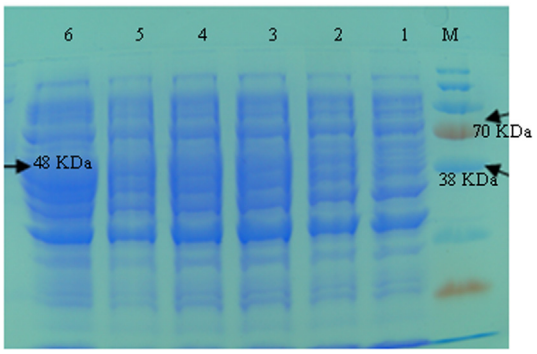

Fig. 3. Analysis CPsV-CP expression level (intensity) through the time course M: pre-stained protein marker. 1–6: the cell lysates after 1, 2, 3, 4, 5 and 16 h post induction.

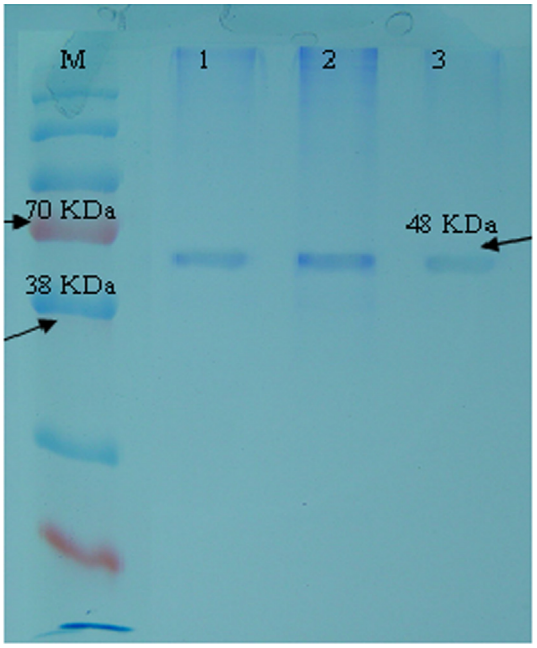

Fig. 4. Shows the Purified expressed coat protein. M: pr-stained protein marker. 1–3: three fractions from the purified expressed coat protein during purification.

was subjected to specificity assay by DAS-ELISA against the commercial purchased antibodies. Diluted CP 1:100, 1:200, 1:400, 1:800 and 1:1600 reacted positively in DAS-ELISA against the commercial purchased antibodies. ELISA values were 1.287, 1.004, 0.654, 0.443 and 0.324 for the above mentioned dilutions respectively and 0.145 for PBS (dialysis and dilution buffer) as a negative control (data collected in Table 2).

3.4. Polyclonal antibodies evaluation

Polyclonal antibodies were raised in mice against the purified CPsV CP fused with 6xHis. The developed polyclonal antisera were evaluated for their specific reaction with CPsV-CP fusion protein (purified and bacterial extract) as well as with CPsV infected plant tissues by ELISA and Western blotting. In ELISA, raised antibodies were reacted positively as trapped antibody with total soluble proteins extracted from CPsV infected citrus tissues and bacterial cells transformed with recombinant plasmid as well as the purified expressed protein. On the other hand the raised antibodies reacted negatively with total soluble proteins extracted from healthy citrus tissues and bacterial cells transformed with non-recombinant plasmid. ELISA results were concluded in Table 3.

Table 2
The specificity of the purified recombinant protein determination by DAS-ELISA against the CPsV monoclonal antibodies specific to the Italian isolate.

Protein dilutions	ELISA detection	
	ELISA value	Result
1:100	1.287	+
1:200	1.004	+
1:400	0.654	+
1:800	0.443	+
1:1600	0.324	+
PBS	0.145	–

The dilutions of the purified recombinant protein (1.5 mg/mL), reacted positively compared to PBS (dialysis and dilution buffer) as a negative control. +: Positive. –: Negative.

The same results were obtained by western blotting analysis. The raised antibodies detected the CP band in total soluble proteins extracted from infected citrus tissues and bacterial cells transformed with recombinant plasmid in addition to the purified expressed protein; conversely there are no detected signals with the total soluble proteins extracted from healthy citrus tissues and bacterial cells transformed with non-recombinant plasmid (Fig. 5).

3.5. ELISA screening using the raised antibodies

To confirm the efficacy of the raised polyclonal antibodies, were evaluated as trapped and intermediate antibodies to screen the 27 citrus samples which previously screened using the commercial antibodies purchased from Agritest, Valanzano, Italy.

CPsV particles were detected in 23 out of 27 samples in both types of ELISA. ELISA reading values showed high efficiency of the raised antibodies as intermediate antibodies in CPsV rather than trapped (Table 4).

Comparing ELISA results obtained by the raised polyclonal antibodies with that previously obtained using the commercial antibodies purchased from Agritest, Valanzano, Italy were recorded (Fig. 6).

4. Discussion

Generally, molecular methods for virus detection such as RT-PCR are not suitable as routine tests for indexing large numbers of samples due to costs and the relative complexity of execution. On the other hand, indexing on woody indicators takes a long time

Table 3
The values obtained for the ELISA of the raised antiserum. The positive reaction with the infected citrus tissue extract and the cell lysate from transformed bacteria with recombinant plasmid as well as the purified recombinant protein and the negative reaction with the healthy citrus tissue extract and the cell lysate from the bacteria transformed with non-recombinant plasmid can be noted.

Infected Citrus sample (+ve)	Healthy Citrus sample (–ve)	Cell lysate transformed with recombinant plasmid	Cell lysate transformed with non-recombinant plasmid	Purified recombinant coat protein
0.654	0.220	0.987	0.230	1.287
+	–	+	–	+

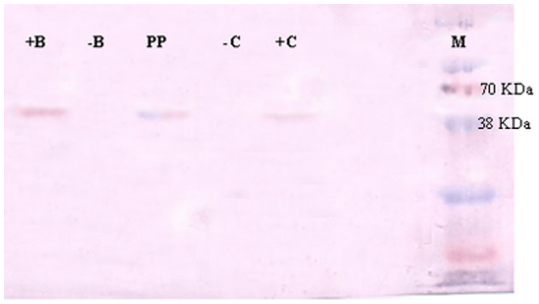

Fig. 5. Western blotting signals developed from the reaction between the raised PAbs and antigens. +C: CPsV-infected citrus plants. +B: transformed bacteria with recombinant plasmid. PP: purified expressed protein. −C: healthy citrus plants. −B: transformed bacteria with non-recombinant plasmid. M: pre-stained protein marker.

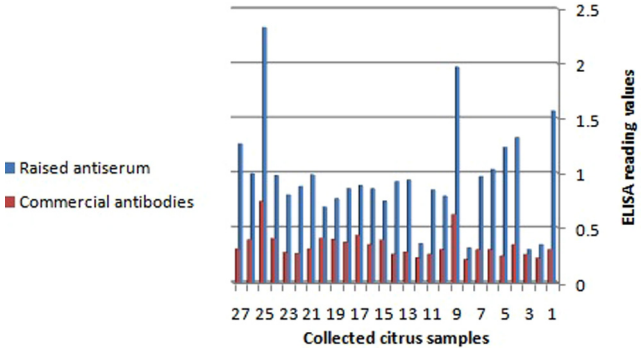

Fig. 6. Comparison between the ELISA results obtained by using the raised and purchased CPsV antibodies.

Table 4
The detection of CPsV infected citrus plants (27 samples) by ELISA, using the raised polyclonal antibodies as intermediate antibodies:

Sample No.	ELISA reading values	Sample No.	ELISA reading values	Sample No.	ELISA reading values
1	1.566	10	0.788	19	0.765
2	0.343	11	0.845	20	0.688
3	0.299	12	0.354	21	0.983
4	1.323	13	0.934	22	0.877
5	1.234	14	0.922	23	0.799
6	1.032	15	0.743	24	0.978
7	0.968	16	0.855	25	2.328
8	0.315	17	0.886	26	0.992
9	1.968	18	0.856	27	1.266
P. control	2.232	N. control	0.311		

for symptoms to be expressed. CPsV is indeed difficult to transmit and maintain on herbaceous hosts, on which it multiplies to low concentrations. Moreover many CPsV isolates are not mechanically transmissible from infected orange to other citrus or herbaceous hosts (Nickel et al., 2004). As a consequence, serology has traditionally been the most used method of plant virus diagnosis in a large number of samples, using ELISA as the method of choice (Djelouah et al., 2002; Scott et al., 2000). In our study, RT-PCR was successfully used to isolate the full length of CPsV-CP gene from infected citrus tissues. The expression of CPsV-CP full length fused with 6xHis tagged was used to obtain the purified and concentrated recombinant fusion protein by affinity purification. Purified expressed coat protein was injected in mice for raising diagnostic polyclonal antibodies for the field virus detection. Such a strategy has been used successfully for a number of plant viruses (Ling et al., 2004; Kamo et al., 2010; Galluzzi et al., 2007; Kumari et al., 2001; Iracheta-Cárdenas et al., 2008; Rubinson et al., 1997). His-tagged CPsV coat protein was expressed in soluble fraction which supported by many authors (Kumari et al., 2001; Nickel et al., 2004; Vaira et al., 1996) but not agreed with others, where their interest fusion proteins were detected in inclusion bodies from transformed *E. coli* as reported for *Faba bean necrotic yellows virus* (Galluzzi et al., 2007), *sugarcane yellow leaf virus* (Hema et al., 2003) and *Groundnut bud necrosis* virus (Jain et al., 2005). Expression results confirmed the presence of expressed CPsV-CP fused with 6xHis in N-terminal (approximately 48 KDa), which matched the deduced amino acids expected by using translation option of Lasergene software analysis for the isolated sequence and previous literatures results.

Since N-terminal His-tag was not expected to have significant immunogenic properties (Kumari et al., 2001), affinity purified CPsV CP fusion protein successfully used to produce useful, diagnostic polyclonal antiserum. Effective Polyclonal antiserum to the fusion protein were produced and evaluated by ELISA and western blotting, and were able to detect CPsV in most of citrus samples, which collected from citrus plants showing citrus psorosis virus symptoms (23 out of 27) on the other hand the commercial purchased antibodies didn't react with most of these collected samples (2 out of 27), which indicated the higher performance of the raised antibodies against the expressed CPsV-CP in comparing with the commercial purchased antibodies, which support the notion that the availability of an antiserum against recombinant viral CP represents an additional and/or alternative tool for manufacturing standardized kits for the serological detection of CPsV. Furthermore the use of recombinant coat proteins eliminates the need to maintain and purify the virus and makes it possible to standardize the process of antigen and antiserum production and the recombinant clone can be stored indefinitely and then used for antigen preparation to ensure the continuous supply of purified coat protein for long-term PAbs production. The evaluation by western blotting proved the successful detection of CPsV by the antiserum raised against the expressed recombinant coat protein in a good quality even in low titer virus without any of non-specific signals which differ from results of non-specificity have been observed with antiserum prepared from fusion proteins of other viruses (Kumari et al., 2001). So far the number of reports indicating effective DAS-ELISA, are limited (Ling et al., 2000; Djelouah et al., 2002; Fajardo et al., 2007), the antiserum developed in this study can be used for a large scale virus diagnostic.

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