IMMUNOLOGICAL COMPARISONS OF POLYCLONAL ANTIBODIES PRODUCED AGAINST THE RECOMBINANT COAT PROTEIN AND VIRAL PARTICLES FOR DETECTING EGYPTIAN BANANA- CUCUMBER MOSAIC *CUCUMOVIRUS*

HANAN A. NOUR EL-DIN

Department of Microbial Molecular Biology, AGERI, ARC, 9 Gamaa St. Giza, 12619, Egypt

Banana is one of the world's most important tropical fruit crop. It is grown both as a staple food and a major cash crop. It is propagated vegetatively through suckers. Successful of banana cultivation is varied, because it is influenced by biotic and abiotic factors. Among the biotic factors, viral pathogens are known to limit the growth and fruit yield of banana (Jeger *et al.*, 1995). Virus borne is considered as major threat due to abundance of insect vectors and easily available alternate hosts. Damage to the crop plants due to virus infection is difficult to assess and losses are estimated to be billion dollars annually (Hus, 2002). CMV is one of the most devastating threats to the banana industry. Because of increased worldwide movement germplasm through seed and propagative material in global trade and agriculture, accurate diagnosis of virus disease in these materials assumes greater importance for national quarantine service to ensure the save movement of germplasm.

CMV has the broadest host range of any known virus, infecting more than 1,000 species of plants and has been found in all parts of the world (Edwardson and Christie, 1991). CMV causing yellow mosaic and stripes on leaves, besides causing leaf distortion along with stunting of banana plant, contribute as a major serious threat for banana cultivation (Niblett *et al*., 1994). CMV causing banana mosaic disease is an important virus, belonging to genus *Cucumovirus* in the family Bromoviridae (Roossinck *et al.*, 1999). It is a positive-sense, single-stranded RNA virus with a tripartite genome. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. An additional subgenomic RNA (RNA 4) derived from RNA 3 (Palukaitis *et al.*, 1992).

For plant viral diseases management, the strategies are directed to prevent virus infection and spreading in the field by eradication the source of infection to control the disease (Haggag and Kazutaka, 2009). Serological or immunological assays have been developed and used successfully for a number of years for plant viral detection. Antibodies are the important reagents in the serological tests such as, ELISA, dot blot and Western blotting analysis, for screening the plant

materials against viruses. The availability of virus-specific antibodies with high purity and sensitivity is the crucial step in plant virus control.

The aim of this study is to make a comparison immunologically between two antibodies Ab/V and Ab/CP through three different serological methods (Dot-blot, Western blotting and TAS-ELISA) to determine sensitivity and specificity of the both Abs for detecting Ban-CMV.

MATERIALS AND METHODS

Amplification, Cloning and Sequencing of CP gene of Ban-CMV

Samples of banana plants showed the characteristic symptoms-like of Ban-CMV were collected from different governorates in Egypt (Giza, El-Behera and El-Qalubia). Total RNA was extracted from 100 mg of the positive-Indirect ELISA leaf sample according to the procedure described in Tripure Isolation Reagent Manual (Roche Diagnostics Coporation, IN, USA) and was used as templates for RT-PCR reaction (QIAGEN, Germany). A pair of primers was designed to amplify the *CP* gene of Ban-CMV as following:

Forward (F) primer:

5'GGGAATTGGATGCATGGACAAATCTGAATC'3.

Reverse (R) primer:

5'GATTGGATCCCGGAATCAGACTGGGAGCA'3.

The protocol of the QIAGEN one step RT-PCR kit was used for RT-PCR.

After amplification the PCR product was analyzed by electrophoresis, the PCR product was purified using QIAX II purification kit (QIAGEN). The purified fragment was ligated into the pGEM-T easy vector (Promega) and then transformed into *E. coli* Top 10 cells. One recombinant plasmid (pCMV1) was subjected to sequence analysis using the F and R M13 universal primers (ABI Prism 310, version 3.2, Genetic Analyzer at AGERI).

Protein expression and antiserum production against Ban-CMV recombinant protein

The fragment of *cp* gene was removed from the sequenced recombinant plasmid pCMV1 by double digestion with *Sph*1 and *Pst*1, then sub-cloned into the bacterial expression vector (PQE-32) previously digested with the same enzymes. The new plasmid (pCMV2) was transformed into bacterial expression host *E. coli* strain M15 ($pREP_4$) strain. After protein expression, the expressed protein was purified by affinity chromatography and analyzed by SDS-PAGE using the protocol described by Laemmli (1970). After purification, the purified 6X His-tagged Ban-CMV CP (1 mg/injection) was injected into rabbits (Two Newzland white rabbits, approximately 35 days old). The first injection was done with complete Freund's adjuvant (1:1v/v) and the four

remaining injections with incomplete Freund's adjuvant (1:1v/v). One week after the last injection, seven weekly bleedings were carried out (25-30 ml/bleeding/animal). Blood samples were allowed to coagulate for 1 hour at 37°C and the centrifuged at low speed (3000 xg/10 min). The IgG fraction was precipitated from the immune serum with saturated ammonium sulphate.

Immunodetection methods

1- Triple Antibody Sandwish-Enzyme Liked Immunosorbant Assay (TAS-ELISA)

The procedure was conducted using two different prepared IgGs specific to ban-CMV, one of them was raised against viral particles of the Egyptian CMV isolate (MPP Lab, AGERI, Giza, Egypt) (Ab/V) and the other was against the purified expressed CP protein (Ab/CP). ELISA was done in the polystyrene plates using the protocol described by Clark and Adam (1977).

2- Western blotting analysis

Western blot immunoassay was performed as described by Towbin *et al*. (1979).

3- Dot blot analysis

Dot blot immunoassay was used for Ban-CMV detection in banana leaves according to the method described by Banttari and Goodwin (1995).

RESULTS AND DISCUSSION

Molecular characterization of Ban-CMV-cp-gene

Positive ELISA sample was subjected to RNA isolation and RT-PCR amplification of Ban-CMV CP gene. As shown in Fig. (1), amplified band of about 650 bp was detected in the positive sample. No amplified product was detected with the healthy banana leaf sample.

The amplified product was confirmed by sequencing and revealed 659 bases (data not shown). The nucleotide sequence of Egyptian (Egy) Ban-CMV *cp* gene was aligned with the nucleotide sequence of nine different overseas isolates of Ban-CMV-*cp* gene database BLAST program (GenBank). The comparison revealed that the similarities were ranged from 26.7 to 98.3% based on the nucleotide sequences with the highest similarity (98.3%) was found between the Ban-CMV-Eg and Ban-CMV-USA isolate (data not shown).

The *cp* gene of the Egy Ban-CMV was subcloned into bacterial expression vector PQE-32 and expressed in bacterial expression host *E. coli* strain M15 ($pREP_4$) strain. SDS-PAGE analysis revealed a protein band with molecular weight of 27 KDa (26 KDa of *cp* gene and 1KDa of 6X His tagged fusion protein) in the recombinant cell. The purified recombinant protein (Fig. 2) was used as an immunogen for producing specific polyclonal antibodies.

Immunodetection methods for Ban-CMV in infected samples

1- TAS-ELISA

TAS-ELISA was used as a diagnostic tool for detecting the presence of Ban-CMV in the collected samples that were collected from different locations in Egypt. The experimental results of TAS-ELISA revealed that both IgGs were able to recognize the presence of Ban-CMV in the tested samples at a dilution I mg/ml. The ELISA values of the positive tested samples, using Ab/V were 0.666, 0.630, 0.625 and 0.720 in compared with the healthy banana plant with value of 0.225 (Table 1), while the ELISA values of the tested sample with Ab/CP were 0.650, 0.615, 0.655 and 0.620 in compared with the healthy banana plant value 0.230 (Table 2). These ELISA-values were after 45 minutes (min.).

ELISA is a suitable technique for plant virus detection (Bashir and Hampton, 1996). ELISA is more useful in field surveys and still considered the most appropriate techniques for screening large numbers of samples and plays an important role in early detection of viral diseases. There are many factors may influence on the sensitivity and reliability of ELISA assay, one of them is the purity and quality of the prepared antibodies. ELISA not need skills and very cost effective.

2- Dot-blot immunoassay

Both antibodies revealed a positive reaction in dots of infected plant tissues for four samples. The colored spots show the sensitivity of both IgGs in detection of Ban-CMV with the two antibodies, but the color was developed faster in the membrane treated with Ab/CP than the membrane treated with Ab/V. No colored signals with the healthy plant tissue as shown in Fig. (3).

Dot blot analysis is simple, relatively inexpensive and the result can scored visually, it is relatively more sensitive and economical in using the different reagents when compared to ELISA method (Rajasulochana *et al*., 2008; Sharma and Misra, 2011). Furthermore, the test of sample extracts can be blotted on the membrane at the field level and then send to the laboratory for further processing. Therefore, a wide potential application of this technique can result in large scale detection of the virus using specific antibody.

3- Western blotting analysis

The results showed that antibodies raised against expressed CP of Ban-CMV recognized bands of the expected size 26 KDa in the protein extract of infected plants. The same band size was observed with the protein extract from the infected plants when probed the membrane with antibody raised against viral particles. No reaction was observed with the extract of healthy plant as shown in Fig. (4).

Western analysis showed that the produced antisera against expressed CP had distinct reactivity profile than that produced against viral particles and the

color was developed faster in the membrane treated with Ab/CP than that treated with Ab/V. However both antisera detected a protein band that has the same mobility. The purified antigen, either viral particles or expressed CP protein, play an important role in producing highly purified antibodies. The purity of produced antibodies was reflected on the accuracy and sensitivity of the immuno-viral detection methods.

In the present study, the result showed that antibodies produced against expressed CP were similar in sensitivity and specificity with the conventional polyclonal antibodies produced against viral particles. Also, the results indicated that fusion CP maintains its native antigenicity and specificity, providing a good source of antigen in preparation of Ban-CMV related antibodies. There are numerous reports of the expression of plant viruses CP in *E. coli* and use as an antigen, instead of virus particles (Abou-Jawdah *et al*., 2004; Fajardo *et al*., 2007; Hema *et al*., 2008; Salimi *et al*., 2012; Shi *et al*., 2013).

We conclude that this technology bears great potential for the serological diagnosis of viruses in many plants and producing antibodies against a recombinant antigen is an advantageous procedure compared to the labor-intensive for virus purification and maintenance of live virus culture. The clone which carry the *cp*-gene can be stored indefinitely, cultured when needed, CP over-expressed and purified in sufficient yield for IgG production. The development of antibodies against expressed CP protein will be played an important role in supporting surveys, certification programs and indexing of banana mother stocks in Egypt.

SUMMARY

There are many immunological methods available for viruses detection. These methods depend on the quality and purity of antibody used in immunodetection. Availability of purified antigen, free from plant debris, will lead to produce highly purified antibody. The purified antibody in immunodetection methods will increase the sensitivity, specificity and accuracy for virus detection. Cucumber Mosaic Virus (CMV) has worldwide distribution and wide host range. Therefore the presence of highly purified antibody will help us in rapid CMV-immunodetection. The main goal in this study is to compare serologically the sensitivity and accuracy of serological methods by using two antibodies. The first, raised against the recombinant expressed coat protein (Ab/CP) and the second produced against the viral particals (Ab/V) of Ban-CMV. ELISA, Dot blot analysis and Western blotting were used as immunodetection methods. In the three serological methods, both IgGs were able to recognize the presence of Ban-CMV in the tested samples at a dilution I mg/ml. However, Western and dot blot analysis were faster in detection of Ban-CMV than ELISA using Ab/CP.

ACKNOWLEDGEMENT

This work was funded by the Egyptian Science and Technology Development Fund (STDF) grant; project ID: 34.

REFERENCES

Abou-Jawdah, Y., H. Sobh, N. Cordahi, H. Kawtharani, G. Nemer, D. P. Maxwell and M. K. Nakhla (2004). Immunodiagnosis of Prune dwarf virus using antiserum production to its recombinant coat protein. Journal of Virological Methods, 121: 31-38.

Banttari, E. E. and P. H. Goodwin (1995). Detection of potato viruses S, X, and y by Enzyme-linked immunosorbent assay on nitrocellulose membrane (Dot-blot). Plant Disease, 69: 202-205.

Bashir, M. and H. O. Hampton (1996). Serological and biological comparisons of Blackeye cowpea mosaic and phytoviruses isolated seedborne in *Vigna unguiculate* L. Wasp. Journal of Phytopathology, 144: 257-263.

Clark, M. F. and A. N. Adams (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology, 34: 475-483.

Edwardson, J. R and R. G. Christie (1991). In: Cucumoviruses (ed). CRC Handbook of viruses infecting legumes, CRC Press. Boca Raton, Florida, p. 293-319.

Fajardo, T. V. M., D. R. Barros, O. Nickel, G. B. Kuhn and F. M. Zerbini (2007). Expression of Grapevine leafroll-associated virus 3 coat protein gene in Escherichia coli and production of polyclonal antibodies. Fitopatologia Brasileira, 32: 496-500.

Jeger, M. J., S. Eden-Green, J. M. Thresh, A. Johanson, J. M. Waller and A. E. Brown (1995). Banana diseases in: Bananas and Plantains. Edited by Gowen, S. published by Chamann and Hall, 317-381.

Haggag, S. Z. and K. Miyataka (2009). Devolopment of rapid, specific and sensitive detection of Cucumber mosaic virus. African Journal of Biotechnology, 8: 751-759.

Hema, M., C. H. V. Subbareddy, H. S. Savithri and P. Sreenivasulu (2008). Assembly of recombinant coat protein of sugarcane streak mosaic virus into potyvirus-ilke particles. Indian Journal of Experimental Biology, 46: 793-796.

Hus, H. T. (2002). Biological control of plant pathogens (viruses). Encyclopedia of Pest Mangement. (ed. By David P) Marcel Dekker, New York, pp: 86.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 227: 680-685.

Niblett, C. L., S. S. Pappu, J. Bird and R. Lastra (1994). Infectious chlorosis, mosaic and heart rot. In: Compendium of tropical fruit disease. APS Press. St Paul, Minnesota. p. 18-19.

Palukaitis P., M. J. Roossinck, R. G. Dietzgen and R. I. B. Francki (1992). Cucumber mosaic virus. Advanced Virus Research, 41: 281-348.

Rajasulochana, P., R. Dhamotharan and P. Srinivasulu (2008). Comparison of Dac-ELISA for the detection of Cucumber Mosaic and Banana Streak Viruses infecting Banana. Journal of American Science, 4: 1545-1003.

Roossinck, M. J., J. Bujarski., S. W. Ding, R. Hajimorad, K. Hanad and S. M. Scott (1999). Tousignant. In: Family Bromoviridae (ed). Virus taxonomy-eight report of the international Committee on Taxonomy of Viruses. Academic Press. San Diego, Calif., p. 923-935.

Salimi, M., M. Shams-Bakhsh and N. Safaie (2012). Expression of the Tomato Yellow Leaf Curl Virus movement protein gene in *Escherchia coli*. Trakia Journal of Sciences, 10: 36-40.

Sharma, K. and R. S. Misra (2011). Molecular approaches towards analyzing the viruses infecting maize (*Zea mays* L.). Journal of General and Molecular Virology, 3: 1-17.

Shi, Y., Y. Yuan, S. Hu, G. Qin-Sheng, L. Shan-Shan and W. Zhen-Yue (2013). Expression of coat protein gene of cucurbit chlorotic yellows virus in *E. coli* and preparation of antiserum. Acta Phytopathologica Sinica, 43: 495-499.

Towbin, H., T. Staehelin and J. Gordon (1979). Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. PNAS, 76: 4350-4354.

Table (1): I-ELISA for evaluation the banana samples, using polyclonal antibodies raised against Ban-CMV particles.

Sample	ELISA detection		Source of samples
	EV	R	
H	0.225	-	Tissue culture sample
1	0.666	+	El-Giza 1
2	0.630	+	El-Behera
3	0.625	+	El-Qalubia
4	0.720	+	El-Giza 2

EV: ELISA values R: Result + : Positive - : Negative

Table (2): I-ELISA for evaluation the banana samples, using polyclonal antibodies raised against expressed CP of Ban-CMV.

Sample	ELISA detection		Source of samples
	EV	R	
H	0.230	-	Tissue culture sample
1	0.650	+	El-Giza 1
2	0.615	+	El-Behera
3	0.655	+	El-Qalubia
4	0.620	+	El-Giza 2

EV: ELISA values R: Result + : Positive - : Negative

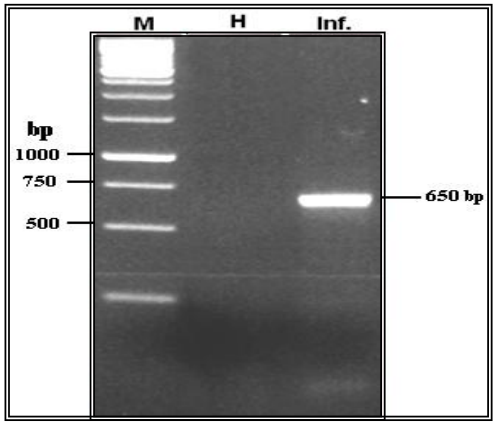

Fig. (1): Agarose gel showing RT-PCR amplification of *cp*-gene of Ban-CMV. M: standard DNA marker. Inf: RT-PCR amplification using RNA from infected tissue. H: RT-PCR amplification using RNA from healthy tissue.

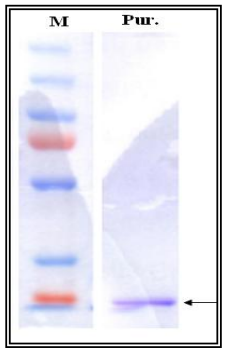

Fig. (2): SDA-PAGE for purified recombinant Ban-CMV CP. M: protein marker. Pur.: purified expressed protein.

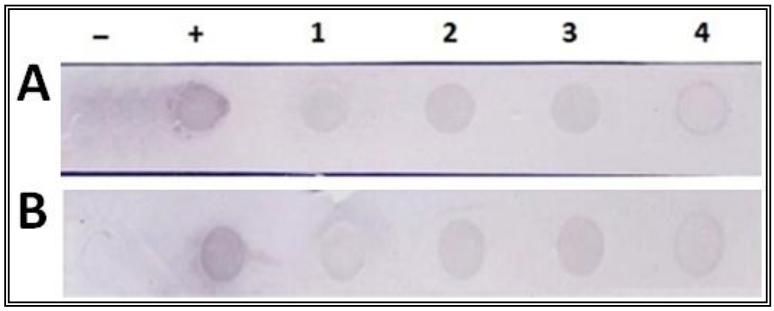

Fig. (3): Dot blot analysis of the extracted proteins from infected banana plants. A: Antibodies against Ban-CMV particles. B: Antibodies against expressed CP of Ban-CMV. (-): healthy tissue sample. (+): Non-infectious positive control. Lanes 1-4: infected tissue samples.

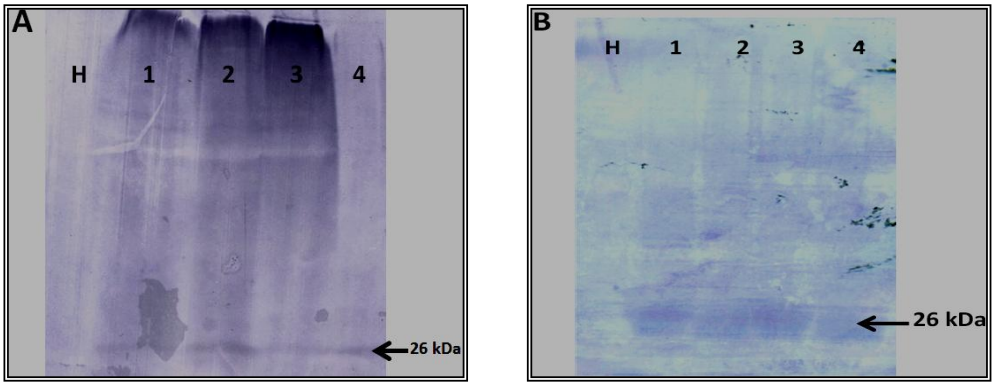

Fig. (4): Western blot immunoassay of the extracted proteins from infected banana plants. A: Antibodies against CMV. B: Antibodies against expressed CP of CMV. Lane H: healthy tissues as a negative control. Lanes 1-4: infected tissue samples.