Egyptian J. Virol. 1, 71-80 (2004)

# Production of Polyclonal Antibodies against the Recombinant *Citrus tristeza virus* Coat Protein Expressed in *Escherichia coli*

**Hala A. Amin[1]; A. Barakat[2]; A.A. Abou Zeid[1]**

*[1] Virus and Mycoplasma Research Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. [2]Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.*

Early diagnosis based on immunoenzymatic techniques requires significant amounts of immunoreagents. Hence, using directional cloning technology, a 38-KDa fusion protein containing a complete coat protein (CP) gene of *Citrus tristeza virus* (CTV) encoded by the expression vector plasmid (PinpointTM Xa3, Promega) with a fragment of biotin binding protein (BBP-rCTV-CP) was highly expressed and purified from *E. coli* cell culture. Antiserum obtained from rabbits after injection with the BBP-rCTV-CP fusion protein showed comparable reactivity in the serological detection of CTV. This antiserum could be used at dilution of 1: 10,000 at the detection stage in an indirect ELISA (IDAS-ELISA) and were efficient for trapping the virus in standard ELISA. These polyclonal antibodies reacted with a wide range of CTV isolates from different Egyptian geographic sources, and of different biological properties.

## INTRODUCTION

*Citrus tristeza virus* (CTV), the causal agent of tristeza disease of citrus, is the most destructive and economically important virus of commercial citrus worldwide (Bar-Joseph *et al.*, 1989 and Lee & Rocha-Peña, 1992). The virus particles, a member of the *closterovirus* group, are flexuous, thread-like and 2000 nm long (Bar-Joseph and Lee, 1990). The genome of this *closterovirus* is single stranded, positive sense, RNA molecule about 20,000 nucleotides organized in 12 open reading frames (ORFs) encoding at least 17 proteins (Karasev *et al.*, 1995; Mawassi *et al.*, 1996 and Vives *et al.*, 1999). The capsid protein is encoded by a 669-nucleotide (nt) open reading frame with a calculated Mr of 25KDa (Sekiya *et al.*, 1991), which is located near the 3' end of the CTV genome (Pappu *et al.*, 1994). Another protein with a molecular mass 27 KDa (P27) is also associated with CTV virions (Febres *et al.*, 1996). Many strains of CTV have been reported worldwide and they found to cause distinct symptoms on different hosts. The host range of CTV is very narrow, and like other *closteroviruses*, in *Citrus spp.* CTV infection has led to decline and death of million citrus trees mostly grafted on sour orange rootstocks (Bar-Joseph *et al.*, 1985). Decline may be in certain cases quick but slow decline for months or years is most frequent. Strains responsible for quick decline or stem pitting symptoms on sweet orange are usually designated as severe strains. Those that do not cause the mentioned symptoms are referred to as mild strains. The virus is associated with phloem (Bar-Joseph and Lee, 1989). Because of this association, virus purification is very difficult and the yields of purified CTV virions are usually low (Bar-Joseph *et al.*, 1985 and Sekiya *et al.*, 1991). Even the best methods may produce CTV preparations that are still contaminated with host components. In addition, the antigen may not maintain its characteristics along successive purifications. Different approach have

been used to overcome these drawbacks, including the use of CTV coat protein (CP), fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels as immunogen (Marco and Gumpf, 1991), cross-absorption of the antisera with healthy plant extracts (Rocha-Peña *et al.*, 1991), and generation of monoclonal antibodies (Mabs) (Permar *et al.*, 1990). The monoclonal antibody MCA13 reacts predominantly with severe (decline inducing and/or stem pitting) strains of CTV from many regions of the world. However, some severe strains from Spain, Israel and California and most mild strains generally fail to react with MCA13 (Permar *et al.*, 1990). However, the risk of the appearance of strains that lack an epitope previously throught to be conserved is real (Kano *et al.*, 1991) and may give rise to false negatives when monoclonal antibodies are used. Thus, polyclonal or mixtures of monoclonal antibodies seem more appropriate for broad-spectrum detection of the virus. Production of antiserum against bacterially expressed CTV coat protein (rCP-CTV) has been previously described (Manjunath *et al.*, 1993; Nikolaeva *et al.*, 1995 and Bar-Joseph *et al.*, 1997). However, recombinant coat protein as an immunogen for antibody production has provided an alternative way to produce polyclonal antibodies.

Up to our knowledge, production of polyclonal antibodies against recombinant CTV-coat protein *via* gene expression strategy has been done in Egypt for the first time in this study.

## MATERIALS AND METHODS

### Virus isolates and plant materials

Samples of commercially grown varieties of citrus trees grafted on sour orange rootstock have been collected from several orchards in Anshas (AN510, AN5 and AN156) and El-Kanater (K1 & K2). Such samples have shown symptoms and/or symptomless. The Mexican lime (*Citrus aurantifolia*, Swingle) seedlings were maintained in insect-free controlled greenhouse conditions for 3-6 months and with day temperature ranging from 24 to 27 °C. These seedlings were used as indicators for the detection of CTV strains by their biological properties. In addition, the isolates were examined by using MCA13 and revealed the presence of the mild (AN510, AN5) and severe (AN156, K1 & K2) strains.

### Virus isolation and purification of the expressed recombinant virus coat protein

The CTV severe Egyptian isolate (CTV-K1) was directly isolated from an infected plant by immunocapture/Reverse transcription – Polymerase chain Reaction (IC/ RT-PCR) and the coat protein gene amplified as described previously by Barakat *et al.* (2002). The resultant PCR product was cloned into pinpoint ™-Xa-3 expression vector in frame with the fragment of biotin- binding protein (BBP) as previously described by Abou Zeid *et al.* (2002). Since large-scale cultures typically are needed for isolating large quantities of the fusion protein. One litre of CTV-CP transformed BL21 *E. coli* culture in LB broth containing 100µg per ml ampicillin and 2µM biotin was grown at 37°C and induced with 100 µM Isopropyl-β- D- thiogalactopyranoside (IPTG). The cells were disrupted by sonication according to the instructions provided by the manufacturer and centrifuged at 10,000Xg for 15 min at 4 °C and the fusion protein was then purified using 3 ml regenerated soft link TM Soft Release Avidin Resin

according to the manufacturer's instruction (Promega, U.S.A.). The purified fusion protein was quantified by the Bradfored dye at $A_{595}$ nm using spectrophotometer. The purified fusion protein was analyzed and separated by electrophoresis in 10 % gradient polyacrylamide gels using the denaturing discontinuous Tris-glycine-SDS system "SDS-PAGE" (Laemmli, 1970)

**Production of polyclonal antibody against recombinant protein**

The BBP-rCTV-CP fusion protein in PBS buffer emulsified with equal amount of incomplete Freund's adjuvant was injected subcutaneously four times at weekly intervals into young healthy New Zealand rabbit at approximately 1.25, 2.5, 3.75 and 5 mg/ml fusion protein to stimulate the induction of CTV-antibodies. Fifteen days after the last injection, the animals were bled at the peripheral ear vein under warm condition and the sera were collected at 2- times. Two-booster injection with BBP-rCTV-CP at 2.5 mg per ml was given 3 weeks after the last primary injection. The titer was evaluated by indirect double antibody sandwich (I-DAS) ELISA.

**Determination of the specificity and the concentration of the antiserum produced**

***a. Western blot analysis for the antiserum***

A small piece (1 cm2) of CTV –infected citrus leaf tissue was chopped into fine pieces with a razor blade. The tissue was then transferred into 1.5 ml microfuge tube containing 0.5 ml 2X western extraction buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). The tissue was further homogenized with an applicator stick in the extraction buffer, boiled for 5 min, vortexed for 15- 20 sec, and centrifuged for 30 sec. A 30 µl aliquot of the extract was loaded into a 12% SDS polyacrylamide gel and separated by electrophoresis. The separated proteins were then transferred to a nitrocellulose membrane using a midi Transblot electrophoretic fast semi-dry transfer cell (Biometra fastblot) as suggested by the manufacturer. Specific CTV-antiserum was applied and the reaction developed as described by Still *et al.* (1991).

***b. Indirect double-antibody sandwich (I-DAS) ELISA***

Indirect double-antibody sandwich (I-DAS) ELISA was performed as described previously by Nikolaeva *et al.* (1995 and 1997). All tests were performed using the I-DAS-ELISA method to examine the detecting ability of the antiserum. Wells of Nunc microtitre plates were coated with a mixture of CTV specific (3CA5 + 3DF1) monoclonal antibodies (diluted 1/2000 in 0.2 M sodium-carbonate buffer, pH 9.6). After an overnight incubation at 4°C, the wells were washed three times with phosphate buffered saline containing 0.1 % Tween 20 (PBST) and loaded with the 100µl sample of plant tissue extract prepared by grinding 1g of fresh citrus bark in 5 ml of PBS buffer. The plates were incubated overnight at 4°C, washed extensively with PBST and loaded with dilutions of the BBP-rCTV-CP antiserum (Crude antiserum was diluted to 1:5, 1:10, 1:25, 1:100, 1:125, 1:625, 1:1000, 1:2000, 1: 3125 and 1:10,000) in PBS buffer. After incubation for 2hr at 37°C, the plates were washed with PBST, incubated with goat anti-rabbit alkaline Phosphatase conjugate (Sigma, 1: 50,000 dilution) for 2 hr at 37°C, and washed extensively with PBST. After loading with the substrate solution, I

mg per ml p-nitrophenyl phosphate (Sanofi), plates were incubated at room temperature for 30 min, and the ELISA reactions were recorded using an ELISA reader at $A_{405}$ nm.

## RESULTS AND DISCUSSION

To produce immunoreagent for routine diagnosis of CTV in field trees using ELISA technique. We resort to prepare the virus antiserum via generate CTV antigen by expressing the coat protein gene in *E. coli* cells as described by several authors (Nagel and Hiebert, 1985; Nikolaeva *et al.*, 1995; Li *et al.*, 1998 and Sequeira & Nolasco, 2002). This has been done for three reasons: First, the CTV is phloem-restricted virus; second, the virus concentration in infected tissue is so low that purification of virus particles is difficult and Thirdly, there is no herbaceous indicator host for the CTV virus rather than the passiflora plant and this could not be found easy in Egypt.

To express the native CTV-coat protein gene in *E. coli*, the full-length P25-coat protein gene (about 700 bp) was constructed in the prokaryotic expression vector Pinpoint ™ Xa-3 under the control of *tac* promoter to create the $p^{PCP}$ plasmid. The sequences of the PCR primers used, were homologous to the 5' and 3' regions of the gene, starting with the second codon for CTV- CP-protein.

It is essentially to point out that the directional cloning approach in the construction of the CP gene expression cassette is more efficient than those applied by previous workers with other viruses: *Papaya ring spot Potyvirus* (Nagel and Hiebert, 1985) *Potyvirus* group (Li *et al.*, 1998) and *Tuberose mild mosaic virus* (Chen *et al.*, 2002). Translation of this clone is initiated at the second amino acid of the CTV-CP gene.

The fusion protein (38 KDa) was simply purified and produced homogenous preparation of the BBP-rCTV-CP fused protein. The yield of the expressed fusion protein, in the used Pinpoint ™ Xa-3 system, was 7.5 mg $L^{-1}$. This was in agreement with Sequeira and Nolasco (2002) but it is very low as compared to 100mg or 280 mg $L^{-1}$ obtained by Manjunath *et al.* (1993) and Nikolaeva *et al.* (1995), respectively for fusion protein containing CP of CTV, although these authors used different systems for expressing the recombinant protein.

Since the expressed plasmids can be stored for long periods and the production of bacterial expressed viral proteins can be used as a good strategy to provide uniform immunogens for antibody production as it avoids laborious virus purification methods and the need for expensive equipments. These results are in accordance to Sekiya *et al.* (1991); Chen *et al.* (2002) and Sequeira and Nolasco (2002). They confirmed that the virus was associated with phloem and because of this association, virus purification was very difficult and the yield of purified CTV virions is usually low and still contaminated with host components. Thus, the recombinant coat protein as an immunogen for antibody production has provided an alternative way to produce polyclonal antibodies. Similarly, Nikolaeva *et al.* (1995) showed that the recombinant virus CP expressed in the bacterial cells have a great potential as an alternative source of antigen for raising specific antibodies to plant viruses and produced in a large quantities and can be manipulated as needed for specific uses.

**Determination of the specificity and the concentration of the antiserum produced.**

***a-Western blot analysis for the antiserum***

To illustrate the specificity of the produced antiserum, we performed an immunoblot analysis for total proteins extracted from CTV- infected and healthy Mexican lime indicator plants (Fig.1). The antiserum raised against BBP-rCTV-CP fusion protein gave a strong specific reaction with the native CTV-CP. A band of approximately 25-KDa was detected from Anshas isolates [mild (AN510) and severe (AN156)] as shown in Tracks A & B respectively, and Kanater severe isolate (K1) Track F. No reaction was observed with the healthy Mexican lime plant (Tracks C, D & E.). The antiserum to BBP-CP fusion protein gave a strong specific reaction with the native CTV-CP band of approximately 25-KDa from CTV-infected plants. The reaction was equally strong for severe and mild strain, suggesting a broad reaction range towards different Egyptian CTV-isolates, in addition to the preferential detection of denatured CTV-CP. This is in accordance with the results obtained by Nikolaeva *et al.* (1995) and Wanitchakorn *et al.* (1997) and Sequeira and Nolasco (2002), who demonstrated that the fusion protein was highly immunogenic and the antibodies produced against an *E. coli* expressed recombinant CTV-CP perform well as detecting antibodies.

***b- Indirect double-antibody sandwich (I-DAS) ELISA***

To confirm the Western blot results, and to study the performance of the antiserum in preliminary experiments, the indirect ELISA was performed. The results in Table (1) show that the BBP-rCTV-CP antiserum (with preliminary dilution 1:500) demonstrated a very good detecting ability of the CTV in extracts of either severe infected plants from Kanater (K1 &K2) and/or symptomless plants (mild) from Anshas (AN5). They gave 1.039, 0.948 and 0.793 absorbance unit at $A_{405}$nm respectively. A parallel assay was done with the purified BBP-rCTV-CP fusion protein in which the immunogen was detected very well with the antiserum giving 1.064-absorbance unit. On the other hand, the absorbance unit of the healthy Mexican lime was 0.283 (Table 1). Generally, the CTV-antiserum demonstrated a broad reaction range towards all CTV-infected plants. A very good result was obtained with CTV-infected Mexican lime (positive control) when our antiserum applied to used in DAS ELISA: giving0.717 absorbance unit.


*Fig. 1:* Western blot analysis of total proteins from CTV-infected and healthy Mexican lime indicator plants using antiserum raised against BBP-rCTV-CP fusion protein. Track A: CTV-mild infected isolate (AN510) being symptomless on lime. Track B: CTV-severe infected isolate (AN156) on lime plants. Tracks C, D & E: from healthy Mexican lime plant. Track F: CTV-infected lime (K1 isolate).

while, the negative limit was 0.292 at $A_{405nm}$. Therefore, the antiserum raised against the 38-KDa-fusion protein could be used only at dilution 1:500, when used as a first antibody in indirect ELISA, but its sensitivity increased sharply by DAS-ELISA.

The I-ELISA also shows highly reaction with the same purified BBP-CP fusion protein preparation that was used as immunogen and that is in agreement with the results obtained by De Sa *et al.* (2000) who found that the lyophilized expressed coat protein would be particularly useful as a positive non- infectious control in serological tests for virus detection. On the other hand, our result was in contrast with Sequeira and Nolasco (2002) results. The authors reported that the same protein preparation that was used as immunogen could be barely detected in I-DAS-ELISA using the homologous antibodies; suggesting that, time and storage of the purified fusion protein, could be acquire a different conformation, hiding the reaction epitopes. However, this contrast in results may be involved due to the difference between the two used fusion-protein purification protocol systems.

Determination the working dilutions of antiserum raised against BBP-rCTV-CP fusion protein through I-DAS-ELISA is shown in Table( 2). Results indicated that the absorbance units at $A_{405nm}$ for the infected sample at least dilution of antiserum ($10^{-4}$) was 0.023 and 0.075, in the first and second bled respectively, while the absorbance unit of the healthy Mexican lime was 0.001 and 0.023 respectively (Table 2), suggesting that the high efficiency of our prepared antiserum. Moreover, positive reactions were recorded between anti CTV- antiserum and all infected tested samples with all dilutions used even with the minimum dilution of antiserum $10^{-4}$ (Table 2).

Results of the indirect double antibody sandwich I-DAS-ELISA (in combination with selected CTV-specific MCAs) revealed that the raised antiserum could be used for detecting CTV infection at dilution of $10^{-4}$. Therefore, the I-DAS –ELISA definitely increased the sensitivity of the raised antiserum. In this manner, the specificity of the raised antiserum increased when it used as a secondary antibody in I-DAS-ELISA, in combination with selected CTV-specific MCAs, as well as in DAS-ELISA in combination with CTV-antibody conjugate (Sanofi). This result was in agreement with Nikolaeva *et al.* (1997) who reported that, mixed coating with different mouse MCAs and several rabbit and chicken polyclonal antisera, with different serological specificities in I-DAS-ELISA, resulted in substantially increased efficiency of the virus antigen trapping by antisera produced against bacterially expressed protein fragments. I-DAS-ELISA increased sensitivity of the CTV detection after optimization of the ratio between conformational and linear antibodies.

Interestingly, our antiserum raised against 38-KDa (BBP-rCTV-CP) fusion protein is successfully used at the trapping stage of ELISA experiments. In addition, it also enables us to prepare an efficient diagnostic kit that is based solely on a single antiserum produced against the expressed protein. Similarly, there are some reports by Vaira *et al.* (1996) and Chen *et al.* (2002) who used other viruses to produce antibodies against expressed proteins, these antibodies may have good ability to trap the viral antigens. On other hand, these results would rule out the report of Bar-Joseph

*et al.* (1997); who reported that antibodies produced against recombinant proteins recognize sequential epitopes rather than conformational ones. This would be due to the incorrect folding of bacterial expressed protein and therefore this kind of approach is not adequate to produce good trapping antibodies.

It is generally accepted that this kind of approach is adequate to produce good trapping antibodies. Nikolaeva *et al.* (1995) showed that the use of CTV coat protein expressed in and purified from an *E. coli* system to raise polyclonal antibodies avoid the production of antibodies against host proteins that may present in the viral preparations.

**Table 1: Indirect ELISA using the BBP-rCTV-CP antiserum**

| Sample | Indirect -ELISA | |
|---|---|---|
| | ER | Result |
| Severe-Kanater isolate (K1) (1/4 g/coating buffer) | 1.039 | +++ |
| Mild-Anshas isolate (AN5) (1/4 g/coating buffer) | 0.793 | ++ |
| Severe-Kanater isolate (K2) (1/4 g/coating buffer) | 0.948 | ++ |
| BBP-rCTV-CP fusion protein | 1.064 | +++ |
| Healthy Mexican lime (1/4 g/coating buffer) | 0.283 | - |

ER: ELISA readings

K: Kanater

-: Negative reaction

CTV: *Citrus tristeza virus*

AN: Anshas

+: Positive reaction

BBP-rCTV-CP: Biotin binding protein-recombinant CTV-coat protein

**Table 2: Determination of series of dilutions of BBP-rCTV-CP-antiserum against infected and healthy samples by ELISA test.**

| Antiserum dilution | ELISA absorbance unit | | | |
|---|---|---|---|---|
| | First bled | | Second bled | |
| | Sap of Infected sample | Sap of Healthy sample | Sap of Infected sample | Sap of Healthy sample |
| 1/5 | 1.484 | 0.207 | 1.135 | 0.430 |
| 1/10 | 1.477 | 0.106 | 1.057 | 0.332 |
| 1/25 | 1.137 | 0.058 | 0.860 | 0.323 |
| 1/100 | 0.710 | 0.024 | 0.605 | 0.194 |
| 1/125 | 0.433 | 0.019 | 0.517 | 0.163 |
| 1/625 | 0.233 | 0.003 | 0.239 | 0.042 |
| 1/1000 | 0.169 | 0.016 | 0.135 | 0.060 |
| 1/2000 | 0.162 | 0.020 | 0.142 | 0.020 |
| 1/3125 | 0.066 | 0.005 | 0.130 | 0.024 |
| 1/10,000 | 0.023 | 0.001 | 0.075 | 0.023 |

## REFERENCES


Abou Zeid, A.A.; Barakat, A. and Amin, H. A. (2002). Expression and Purification of the Coat Protein gene (P25) of *Citrus tristeza virus* in *E.coli*. Az. J. Microbiol. 58: 1-9.

Barakat, A.; Abou Zeid, A.A. and Amin, H. A. (2002). Immunocapture. Reverse Transcription Polymerase Chain Reaction (IC/RT-PCR) Detection of *Citrus tristeza virus* in Egypt. Az. J. Microbiol. 58: 10-20.

Bar-Joseph, M., and Lee, R.F. (1989). *Citrus tristeza virus*. AAB descriptions of plant viruses, No.353. Commonwealth Mycological Institute/Association of Applied Biologists, Wellesbourne, Warwick, England.

Bar-Joseph, M. and Lee, R.F. (1990). *Citrus tristeza virus*. Description of plant viruses No. 353. Commonwealth Mycological Institute/Association of Applied Biologist. Key Surrey, UK, pp 7.

Bar-Joseph, M.; Marcus, R., and Lee, R.F. (1989). The continuous challenge of *Citrus tristeza virus* control. Ann. Rev. Phytopathol. 27: 291-316

Bar-Joseph, M.; Gumpf, D.J.; Dodds, J.A.; Rosner, A. and Ginzberg, I. (1985). A simple purification method for *Citrus tristeza virus* and estimation of its genome size. Phytopathology 75: 195-198.

Bar-Joseph, M.; Filatov, V.; Gofman, R.; Guang, Y.; Hadjinicolis, A.; Mawassi, M.; Gootwine, E.; Weisman, Y.; Malkinson, M. (1997). Booster immunization with a partially purified *Citrus tristeza virus* (CTV) preparation after priming with recombinant CTV coat protein enhances the binding capacity of capture antibodies by ELISA. J. Virol. Methods 67(1): 19-22.

Chen, C.C.; Hsiang, T.; Chiang, F L.; Chang, C.A. (2002). Molecular characterization of *Tuberose mild mosaic virus* and preparation of its antiserum to the coat protein expressed in bacteria. Bot.Bull. Acad. Sin. 43: 13-20.

De Sa, P.B.; Hiebert, E. and Purcifull, D.E. (2000). Molecular characterization and coat protein serology of *Watermelon leaf mottle virus* (*Potyvirus*). Archive of virology 145: 641-650.

Febres, V.J.; Ashoulin, L.; Mawassi, M. (1996). The p27 protein is present at one end of *Citrus tristeza virus* particles. Phytopathology 86: 1331-1335.

Kano, T.; Garnsey, S. M.; Koizuni, M. and Permar, T.A. (1991). Serological diversity of field sources of *Citrus tristeza virus* (CTV) in Japan. In: Proceeding of the 11th International Organization of Citrus Virology. (R.H. Brlansky, R.F. Lee and L.W. Timmer ed.). IOCV, Riverside, CA, USA, 51-55.

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O.V., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., Lee, R.F., Garnsey, S.M., Lewandowski, D.J. and Dawson, W.O. (1995). Complete sequence of the *Citrus tristeza virus* RNA genome. Virology 208, 511-520.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lee, R.F. and Rocha-Peña, M.A. (1992). *Citrus tristeza virus*. In: Kumar, J., Chaube, H.S., Singh, U.S. and Mukhopadhay, A.N. (eds). Plant Diseases of International Importance. Diseases of Fruit Crops. Prentice Hall, Englewood Cliffs, NJ, Vol. 3: pp 456.

Li, R.H.; Zettler, F.W.; Purcifull, D.E. and Hiebert E. (1998). The amino acid sequence homology of coat proteins as a basis for identification and classification of the *potyvirus* group. J. Gen. Virol. 69: 2710 – 2710.

Manjunath, K.L.; Pappu, H.R.; Lee, R.F.; Niblett, C.L. and Civerolo, E.L. (1993). Studies on the coat protein genes of four isolates of *Citrus tristeza Closterovirus* from In-dia: cloning, sequencing and expression. In: proceedings of the 12th International Organisation of Citrus Virology. (Moreno P., da Graca J.V., and Timmer L.W. ed.), IOCV, Riverside, CA, USA, 20 – 27.

Marco, G. and Gumpf, D.J. (1991). A simple technique for the production of highly specific polyclonal antisera for *Citrus tristeza virus*. In Proc. Conf. Int. Organ. Citrus Virol., 11th, R.H. Brlansky, R. F. Lee and L.W. Timmer, eds. IOCV, Riverside, CA, P 77-81.

Mawassi, M.; Mietkiewska, E.; Gofman, R.; Yang, G. and Bar-Joseph, M. (1996). Unusual sequence relationships between two isolates of *Citrus tristeza virus*. J Gen Virol 77 (Pt 9): 2359-2364.

Nagel, J. and Hiebert, E. (1985). Complementary DNA cloning and expression of the *Papaya ringspot Potyvirus* sequences encoding capsid protein and a nuclear inclusion-like protein in *Escherichia coli*. Virology 143: 435-441.

Nikolaeva, O.V.; Karasev, A.V.; Gumpf, D.J.; Lee, R.F. and Garnsey, S.M. (1995). Production of polyclonal antisera to the coat protein of *Citrus tristeza virus* expressed in *Escherichia coli*: Application for immunodiagnosis. Phytopathology 85: No 6, 691-694.

Nikolaeva, O.V.; Karasev, A.V.; Powell, C.A.; Garnsey, S.M. and Lee, R.F. (1997). Modulation of the antigenic reactivity of the *Citrus tristeza virus* coat protein. J. Immunol. Methods 7, 206(1-2): 97-105.

Pappu, H.R.; karasev, A.V.; Anderson, E.J.; Pappu, S.S.; Hilf, M.E.; Febres, V.J.; Eckloff, R.M.G.; McCaffery, M.; Boyko, V.; Gowda, S.; Dolja, V.V.; Koonin, E.V.; Gumpg, D.J.; Cline, K.C.; Garnsey, S.M.; Dawson, W.O.; Lee, R.F. and Niblett, C.L. (1994). Nucleotide sequence and organization of eight 3' open reading frames of the *Citrus tristeza Closterovirus* genome. Virology 199. 35-46.

Permar, T.A.; Garnsey, S.M.; Gumpf, D.J. and Lee, R.F. (1990). A monoclonal antibody which discriminates strains of *Citrus tristeza virus*. Phytopathology 80: 224-228.

Rocha-Peña, M.A.; Lee, R.F. and Niblett, C.L. (1991). Development of a dot- immunobinding assay for detection of *Citrus tristeza virus*. J. Virol. Methods 34:297-309.

Sekiya, M.E.; Lawrence, S.D.; McCaffery, M. and Cline, K. (1991). Molecular cloning and nucleotide sequencing of the coat protein gene of *Citrus tristeza virus*. J. Gen. Virol. 72: 1013-1020.

Still P.E.; Hunter T.J.; Rocha-Peña M.A.; Lee R.F. and Niblett C.L. (1991). Western blotting as a rapid method for the immuno detection and classification of *Citrus tristeza virus* isolates. (Abstr). Phytopathology citrus tristeza virus 81: 695.

Sequeira, Z. and Nolasco, G. (2002). Bacterial expressed coat protein: development of a single antiserum for routine detection of *Citrus tristeza virus*. Phytopatol. Mediterr. 41, 1-8.

Vaira, A.M.; Vecchiati, M.; Masenga, V. and Accotto G.P. (1996). A polyclonal antiserum against a recombinant viral protein combines specificity with versatility. J. Virol. Methods 56: 209 – 219.

Vives, M.C.; Rubio, L.; López, C.; Navas-Castillo, J.; Albiach-Marti, M.R.; Dawson, W.O.; Guerri, J.; Flores, R. and Moreno, P. (1999). The complete genome sequence of the major component of a mild *Citrus tristeza virus* isolate. J. Gen. Virol. 80: 811-816.

Wanitchakorn, R.; Harding, R.M. and Dale, J.L. (1997). *Banana bunchy top virus* DNA-3 encodes the viral coat protein. Archive of Virol. 142: 1673-1680.