DEVELOPMENT OF LATEX AGGLUTINATION TEST FOR RAPID DETECTION OF CITRUS TRISTEZA CLOSTEROVIRUS

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Citrus is one of the most widely grown and economically important fruit crops in the world for generation of income, foreign trade and nutrition. Citrus Trestiza Virus (CTV) is the causal agent of the most economically important viral disease of citrus worldwide (Bar-Joseph *et al*., 1989; Rocha-Pena *et al*., 1995). CTV, a member of the Closteroviridae, genus *Closterovirus*, has a ~20 kb single stranded, positive sense RNA genome (Karasev *et al.*, 1995). The CTV genome consists of 12 open reading frames (ORFs) and potentially encoding at least 19 protein products. The CTV virons are polarly coated with two separate coat proteins (CPs) p25 and p27, elaborated as major and minor CPs,

respectively (Febres *et al*., 1996). The minor CP is associated with small amount of two other proteins p65 and a large protein p61. The 12 ORFs of CTV are expressed through different number of ways including, proteolytic processing of polyprotein, translational frame shifting and up to 32 different 5´- and 3´-subgenomic RNAs (Che *et al*., 2003). CTV causes different disease syndromes on citrus plants depending on the virus strain. Different CTV strains, generally referred to as seedling-yellows (CTV-SY), tristeza (CTV-T), stem-pitting (CTV-SP), and a mild type, have been widespread for many years. Any of these strains may exist in a citrus plant or they may occur together as a complex (More-

no *et al.*, 2008). The virus has numerous isolates differing in biological (symptoms and aphid transmissibility) and serological properties (Ballester-Olmos *et al.*, 1993; Cambra *et. al.*, 1993; Roistacher and Bar-Joseph, 1984) and genome sequence (Karasev *et al.*, 1995; Vives *et al.*, 1999). The control of this economically important virus is achieved by preventive methods such as quarantine, use of disease free bud wood and CTV-tolerant rootstocks. Eradication programs and the use of cross-protection strategies with mild isolates can also contribute to the control of the disease (Roistacher and Moreno, 1991). All these control strategies require a large number of detection tests usually performed by enzyme-linked immunesorbent assays (Clark *et al*., 1976; Clark and Adams, 1977) and PCR-based assays (Cambra *et al*., 2006). This report describes the development of latex agglutination test (LAT) as rapid, sensitive and reliable method and its application in CTV monitoring in citrus plants.

MATERIAL AND METHODS

Samples collection and virus detection

Leaf samples from citrus trees which have virus-like symptoms were collected from Qaha, El-Kalubia governorate. DAS-ELISA was carried out using ELISA Kit (Agritest Co., Italy) according to manufacturer's instructions.

Amplification of CP gene (p25) of CTV by RT-PCR

Total RNA was extracted from 50-100 mg of positive-ELISA leaf samples according to the procedure described in Tripure Isolation Reagent Manual (Roche Diagnostics, USA). The extracted RNA was used as a template in the RT-PCR reaction (Qiagen, Germany) and the following specific primers were used for *p25* gene amplification:

Forward primer (CTV-F):
5′GCG<u>GGATCC</u>ATGGACGACGAAACAAAGAAATTGA′3

Reverse primer (CTV-R):
5′CGA<u>AAGCTT</u>TCAACGTGTGTTGAATTTCCCA′3

The underline letters represent *Bam*HI and *Hind*III restriction sites in the CTV-F and CTV-R primers respectively. After amplification, PCR products were separated by electrophoresis in a 1.2% agarose gel and detected by ethidium bromide staining.

Cloning, sequencing and expression of CTV-p25 gene.

RT-PCR product was cloned into pGEM-Teasy (Promega, USA) and the nucleotide sequence was determined in both directions with an ABI Prism DNA sequencer 377 (Applied Biosystems, USA). The PCR product was subcloned into a protein expression vector pQE-30 (Qiagen, Germany), which tags the expressed protein with N-terminal hexahistidine (6xHis tag), allowing easier purification by affinity chromatography. After purification, the recombinant protein was analyzed by SDS-PAGE (Laemmli, 1970).

Production and purification of polyclonal antibodies against CTV-p25 recombinant protein

The purified recombinant protein was used for preparing antibodies in New Zealand white rabbits. The rabbit was first immunized subcutaneously with 500 μg recombinant protein in Freund's complete adjuvant. Two booster injections were given with 250 μg recombinant protein each in incomplete Freund's adjuvant at 3-weeks interval, and the antiserum was collected 7 days after the last boost. The rabbit IgG fraction was precipitated from the immune serum with 50% saturated ammonium sulphate and purified by DEAE–Sepharose column chromatography (Clark and Adams, 1977).

Coating of Latex particles with the CTV-p25 antibodies

The mechanism of covalent coupling of antibodies by activating the carboxyl groups with water-soluble carbodiimide is illustrated in Fig. (1). Latex particles and the PolyLink Protein Coupling Kit for carboxylated Microspheres were purchased from Bangs Laboratories, Inc. (USA). The purified antibodies were coupled to carboxylated latex particles (0.79 μM) according to the manufacturer's instructions. Briefly, microparticles (12.5 mg) were pipetted into 1.5 ml microcentrifuge tube and pelleted by centrifugation for 10 min at 5000 rpm. Microparticle pellet was resuspended in 400 μl of polylink coupling buffer (50 mM MES, pH 5.2; 0.05% Proclin® 300) and repelleted for 10 min at 5000 rpm.

The resulted pellet was resuspended in 170 μl of polylink coupling buffer. Just before use, 200 mg/ml EDAC solution (Carbodiimide) was prepared by dissolving 10 mg polylink EDAC in 50 μl polylink coupling buffer. EDAC solution (20 μl) was added to the microparticles suspension and mixed gently end-over-end or briefly vortex. The produced antibodies were added in concentration of 500 μg and mixed gently and incubated 1 hr at RT with gently mixing. After 1 hr the mixture was centrifuged for 10 min at 5000 rpm. The supernatant was discarded and the microparticle pellet washed twice with polylink wash/storage buffer (10 mM Tris, pH 8.0; 0.05% Bovine Serum Albumin; 0.05% Proclin® 300). Finally the particles were resuspended in 400 μl of polylink wash/storage buffer and stored at 2-8°C until use.

Latex agglutination test

Infected and healthy tissues of citrus plants were extracted with 0.05 M Tris-HCl, pH 7.2, containing 0.02% PVP (Omura et al., 1984). The extracts were mixed well in equal volumes (50 μl) with the coated latex particles. The mixture was incubated at room temperature from 10 min and the agglutination was observed visually.

RESULTS AND DISCUSSION

Molecular characterization of CTV-CP gene

The DAS-ELISA values confirmed the presence of CTV in some tested sam-

ples and the values were 2-3 folds of the healthy citrus sample. Three samples out of 20 revealed positive reaction with the polyclonal antibodies. Positive ELISA values were 0.595, 0.6514 and 0.750, while the mean value of the healthy samples was 0.2. One of the positive ELISA samples (0.75) was subjected to RNA isolation and RT-PCR amplification of CTV-CP gene (*p25*). As shown in Fig. (2), a detectable band of ~690 bp was recognized from RT-PCR of the positive sample. While RT-PCR for the healthy sample revealed no amplified product. The identity of the amplicon was confirmed by sequencing and revealed 672 bases (data not shown). DNA sequence of the putative CTV-CP was compared to those in public database BALST program (GenBank). The comparison revealed a high degree of similarity up to 98% with others CTV-CP gene from different isolates. After that, P25 coat protein gene was subsequently subcloned into bacterial expression vector pQE-30 and expressed in Escherichia coli BL21 (DE3) strain. The expressed P25 coat protein was analyzed by SDS-PAGE in which the presence of a band with a molecular mass corresponding to approximately 26 kDa, an expected value for the fusion protein, was observed (Fig. 3). The purified recombinant protein was used as an immunogen for producing polyclonal antibodies for coating latex particles.

Latex agglutination test

The coated latex particles with CTV-CP antibodies were mixed with the sap of the infected and healthy plants. As shown in Fig. (4), positive result showed the development of an agglutinated pattern resulting from the clumping of the latex particles. Negative result showed no agglutination. The identification, detection and diagnosis of plant viruses rely on biological, serological, and nucleic acid-based techniques. Serological techniques, which include ELISA (Clark and Adams, 1977), tissue blot immunosorbent assay (Lin *et al*., 1990), are powerful tools for the detection of plant viruses. Effective strategies for controlling CTV or any other plant viruses is depend on sensitive and rapid diagnostic methods. Bioassay, electron microscopy, nucleic acid-based techniques or serological tests have been used in identify viral infection in the plants. ELISA and immunosorbent electron microscopy (ISEM) have been widely used for diagnosing many viruses as sensitive and reliable methods. The major disadvantages of these methods are the time required to complete the test and the associated expenses. Also, these methods are laborious to analyze a large number of samples. Latex agglutination method has advantages over these methods, because it does not require specialized equipment and it can be done in easy manner with considerably high sensitivity. In this study we developed latex beads coupled with antibodies produced against the recombinant expressed P25 coat protein of CTV. This method should improve diagnostic capabilities in developing countries, as a large number of samples during surveys of large areas can be tested in the field or any primitive laboratory within minutes. It is clear evidence indicating that no false-

positive signals were given from the healthy plants, although non-specific reactions were the main problem in some diagnostic methods as reported by some authors (Hibi and Saito, 1985; Powell, 1987; Hus *et al.*, 1992). These results are in agreement with the results obtained by several authors (Talley *et al.*, 1980; Fribourg and Nakashima, 1984; Demski *et al.*, 1986; Sijam and Sulaiman, 2003). An interesting fact about latex agglutination test is that latex particles can be coated with different antibodies produced against different viruses so that we can perform the test for the diagnosis of different plant viruses at the same time. Furthermore, agglutination reactions employing synthetic beads can be read rapidly, often within 5 to 10 minutes of mixing the beads with the test sample. This technique can be used in routine testing by grower's advisory services for certification of healthy stocks. Finally as noted above, latex test is a sensitive, simple, rapid and reliable procedure for diagnosis of several plant viruses or any other plant pathogen. The simplicity of the detection method makes it economically acceptable and technically adopted as the antigen can be prepared with limited chemical and equipment.

SUMMARY

Reliable, simple, and rapid detection tests are needed to better monitor and manage plant virus infection. Microsphere-based diagnostic tests can fulfill these needs and are inexpensive and portable for any laboratory. In the present study, a latex agglutination test (LAT) using antibody-labeled latex beads for detecting Citrus tristeza virus (CTV) was developed. Polyclonal antibodies were immobilized on the surface of latex beads and tested for the ability to detect CTV. Under optimized conditions, LAT revealed successful detection of the viral particles in the infected citrus plant.

ACKNOWLEDGEMENT

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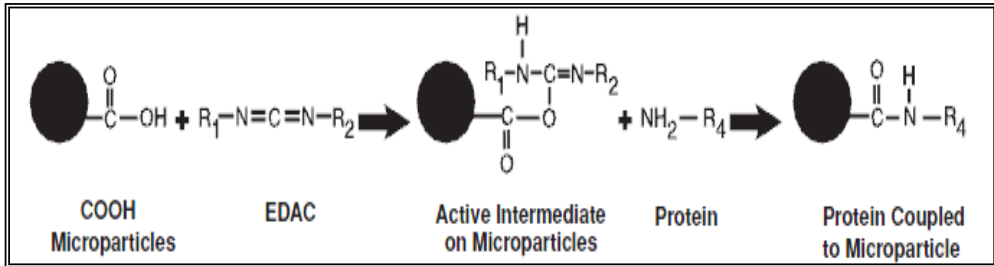
Fig. (1): Mechanism of coupling protein (antibodies) with activated Carboxyl (COOH) microparticles.

Fig. (2): RT-PCR amplification of putative CTV-CP gene (p25). M: 100 bp ladder. Lanes 1 and 2: RT-PCR products from healthy and infected citrus plants, respectively.
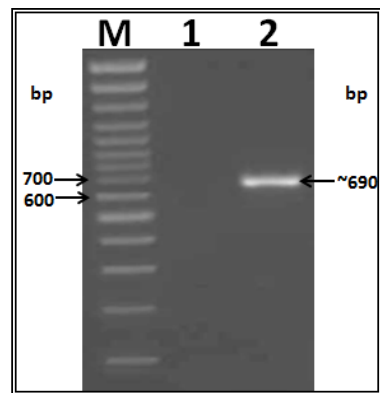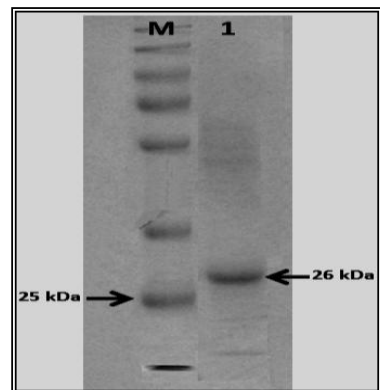

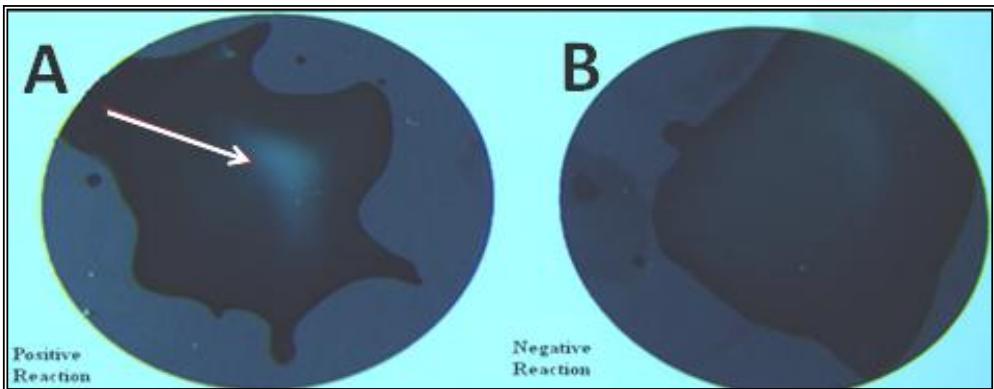Fig. (3): SDS-PAGE for purified recombinant CTV-CP. M: protein marker. Lane 1: purified expressed CTV-CP.

Fig. (4): Latex agglutination test for detecting CTV in citrus plants. A: positive reaction with infected citrus plant with CTV, arrow indicates agglutination. B: negative reaction with healthy citrus plant.