Detection of plants infected with citrus tristeza virus using serological methods in northern and southern regions of Iran

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ABSTRACT

Objective: Citrus tristeza virus is economically one of the most important citrus diseases in Iran. To prevent the impact of the disease in citrus growing areas, the production of virus-free plants and the removal of infected plants are key methods. In line with these goals, it is very important to obtain simple and sensitive diagnostic tools. The main goal of the present study is to develop serological methods for efficient detection of Tristeza virus in citrus cultivation areas.

Methods:Text For this purpose, the gene encoding the coat protein (CP25 KDa) of Citrus tristeza virus was expressed in bacteria and purified through affinity chromatography. For the production of specific antibody against Tristeza virus, purified recombinant envelope protein was used to immunize rabbits, and then purification of immunoglobulin was done using protein A column. Antibodies and conjugates were made and purified, suspected plants with symptoms of citrus tristeza virus infection were collected from different parts of Mazandaran and Kerman province, ELISA, western blot and DIBA serological tests were used to identify infected plants.

Results:Text The results showed that the produced antibodies can be effectively used to identify plants infected with citrus tristeza virus, and out of 51 samples suspected of virus contamination, fourteen plants were definitely diagnosed with a high percentage of infection."

Conclusions: The use of the antibody produced to identify the infected plants collected from the north and south of Iran shows gave that it can be effectively used for different isolates of Triestzai virus released in different regions of the country.

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1.Introduction

Citrus tristeza virus (CTV) is distributed worldwide and is the cause of one of the most important citrus virus diseases (Rocha-Pena *et al*., 1995a). This virus is transmitted semi-permanently by aphids (Bar-Joseph and Lee, 1989). The virus has a long, flexible, filamentous body (2000 nm in size) and a positive single-stranded RNA genome that contains 19,296 nucleotides organized into 12 open reading frames and encodes at least 19 proteins (Lee and Bar-Joseph, 2000; Niblett *et al*., 2000). The RNA genome is enclosed by two coat proteins, one is 25 kDa and the other is 27 kDa, designated as major coat protein (CP) and minor coat protein (p27), respectively (Febres *et al.,* 1996; Lee and Bar -Joseph, 2000). Citrus tristeza virus has a large number of distinct strains that cause very different symptoms in different citrus species and cultivars (Bar-Joseph and Lee 1989; Garnsey *et al*., Niblett *et al.,* 2000). Tristeza disease entered Iran with the arrival of tangerine seedlings (Citrus unshiu) from Japan, which were planted in Mahdasht gardens in Mazandaran province, and it was reported from Iran between 1968 and 1970 (Bove, 1995). The use of serological methods, mainly different forms of ELISA (Bar-Joseph *et al.,* 1977; Bar-Joseph and Malkinson, 1980; Rocha-Pena *et al.,* 1991a; Garnsey *et al*., 1993)) has become an essential tool for Large-scale detection of citrus tristeza has become widespread worldwide for research and virus control purposes (Bar-Joseph *et al.,* 1979; Garnsey *et al.,* 1981; Bar-Joseph and Lee, 1991; Rocha-Pena *et al*., 1993; Mathew *et al.,* 1997; Cambra *et al.,* 2000). Production of antiserum for the detection of Tristeza virus, either in the form of polyclonal antibodies (Gonsalves *et al*., 1978; Bar-Joseph and Malkinson, 1980; Marco and Gumf, 1991). or as monoclonal antibodies (Vela *et al.,* 1986; Permar *et al.,* 1990; Ozturk and Cirakoglu, 2003). It was usually done in the past using purified virus. Production of polyclonal antibodies requires large quantities of purified virions for use in immunization procedures. Production of polyclonal antibodies requires large quantities of purified virions for use in immunization procedures. The use of the Tristeza virus envelope protein expressed in the Escherichia coli bacterial system, which has been purified and used to produce polyclonal antibodies, overcomes these limitations and avoids the production of antibodies against host proteins that may be present in viral preparations. (Nikolaeva *et al.,* 1996). The generation of antiserum against the bacterially expressed envelope protein of citrus tristeza virus (rCP-CTV) has been previously described (Sequeira *et al*., 2002, Manjunath *et al*., 1993, Nikolaeva *et al*., 1995, Bar-Joseph *et al*. , 1997.). Sequencing of the coat protein gene of the virus was carried out from the Tristeza isolates in northern Iran (Alavi, V., *et al*., 2005), Tristeza virus specific antisera against the recombinant coat protein expressed in the bacteria were produced to detect the Tristeza virus (Manjunath *et al.* 1997, 1993; Nikolaeva *et al*., 1995; Targon *et al.,* 2002, Bar-Joseph *et al.,* 1997). This method has been used to produce specific antibodies against several plant viruses (Shams-Bakhsh *et al.,* 2004, Cerovska *et al*., 2010, Iracheta *et al*., 2008). the identification of infected plants in the northern and southern regions of Iran was done well by the specially produced serological kit. The use of the Tristeza virus envelope protein expressed in the Escherichia coli bacterial system, which has been purified and used to produce polyclonal antibodies, overcomes these limitations and avoids the production of antibodies against host proteins that may be present in viral preparations. (Nikolaeva *et al.,* 1996). Determining the sequence of the viral coat protein gene from the isolates of Tristeza in the north of Iran was carried out (Alavi, V., *et al.,* 2005). This method has been used to produce specific antibodies against several plant viruses (Shams-Bakhsh *et al*., 2004, Cerovska *et al*., 2010, Iracheta *et al*., 2008).

2.Material and Method

2.1. Expression and purification of envelope protein

The bacterial expression vector containing the coat protein coding gene, pET28a-CP, made in Royan Research Institute by Dr. Hosseini Salekdeh for the production of recombinant coat protein, was provided to this research (Barzegar *et al*., 2005). Plasmid transfer to E. coli strain BL21 led to the production of recombinant coat protein with histidine end. To produce protein, a colony of the above bacteria was cultured in LB medium containing 25 µg/ml of kanamycin. To induce protein expression, IPTG was used with a concentration of one millimolar, then the recombinant protein was purified by affinity chromatography (IMAC) using Qiagen's Ni-NTA

resin column. In order to check the amount of purified protein, Leamli's electrophoresis method was used in the presence of sodium dodecyl sulfate along with specific concentrations of the standard protein bovine serum albumin (BSA) (Leammli *et al*., 1970). The quality of the extracted protein was evaluated using western blot analysis. For this purpose, SDS-PAGE electrophoresis was first performed for the purified envelope protein, then the separated bands were transferred to nitrocellulose membrane, blocking was done with 2% skim milk for two hours, then with primary antibody (anti-His tag) with One-to-thousand dilutions were covered. Then the secondary antibody conjugated with alkaline phosphatase was used with a dilution of one to three thousand.

2.2.Antibody preparation

Two New Zealand white rabbits were used for immunization. Injections were performed intramuscularly at intervals of two weeks, each injection contained 100 µg/ml of dialyzed recombinant protein and the same volume of Freund's incomplete adjuvant (Yinghai *et al.,* 2007). Indirect ELISA was performed to determine the titer of polyclonal antibodies. After completion of the immunization period and blood sampling in order to determine the titer of antisera obtained from rabbits, indirect ELISA assay was performed in 96 plates. Maxisorp™ 96-wells microtiter plate (Nunc-Immuno™, Denmark) coated with recombinant protein was performed (Yinghai *et al*. al., 2007). Different dilutions of 1:512 to 1:262144 dilutions were prepared from the resulting sera with 1x PBS phosphate buffer. In each well of the ELISA plate, 100 microliters of antigen prepared with a concentration of 10 micrograms per milliliter was poured and placed overnight at four degrees Celsius. Then the wells were blocked with 2% skim milk. Then the antisera were added to the wells in the amount of 100 microliters in different dilutions and placed at 37 degrees Celsius for two hours. After washing three times, the bound antibodies were identified by the GARAP secondary antibody after the addition of para-nitrophenyl phosphate precursor. The ELISA plate was placed at a temperature of 37 degrees Celsius for 15 to 30 minutes, and then the amount of light absorption at 405 nm was recorded by an ELISA reader (Teca, otrish). In all stages, washing was done three times with phosphate-buffered saline-Tween (Tween 20%, 0.05%). The obtained serum was stored at -20 degrees Celsius. Purification of antibody from serum was performed using a column containing protein A according to the manufacturer's instructions (AbD serotec, UK). The concentration of purified antibody was estimated using SDS-PAGE. In order to use the antibody prepared in the ELISA method, the purified antibody was attached to alkaline phosphatase (AP) and HRP enzymes. Purified antibodies were labeled with alkaline phosphatase enzyme according to the instructions of the AbD serotec, UK kit (Nakane and Kawaoi, 1974). The produced labeled antibody was used to detect citrus tristeza protein in infected plant tissues in ELISA and dot blot test.

2.3.Examining diseased plant samples from northern and southern regions of the country

51 plant samples, including branches, leaves and stems, were obtained from citrus orchards in the north and south of Iran, respectively, from the cities of Sari, Babolsar, Nowshehr, Ramsar and Noor from Mazandaran province and Jiroft from Kerman province. The samples include orange, sweet orange, lime trees. which had the symptoms of chlorosis, lightness of veins and red cracking (Figure 1). First, contamination of plant samples with Tristeza virus using a commercial citrus Tristeza virus ELISA kit (Bioreba, Switzerland) was confirmed, then the presence of Tristeza virus in plant samples was evaluated by DAS-ELISA using the antibody and conjugate prepared in this research. For this purpose, the wells were filled with a polyclonal antibody against the pure coat protein with a dilution of 1:000 and incubated at 37 degrees Celsius for 2 hours. Plant extract was prepared with extraction buffer (Tris buffer pH7.4 containing 137 mM NaCl, 3 mM KCl, 2% PVP 24 kDa, 0.05% Tween20 and 0.02% NaN3). Plant extracts and pure recombinant protein (positive control) were added to the plate and incubated overnight at 4 degrees Celsius. Then polyclonal antibody bound with AP and HRP was added at a dilution of 1:1000 and incubated at 37°C, absorbance values were read at 405 nm after thirty minutes. Samples that absorbed at least twice as much as the healthy plant sample were considered positive (positive and negative control samples respectively included plant samples infected with Tristeza virus and healthy plants whose contamination was confirmed by commercial ELISA kit). The accuracy of the results was reassessed using a commercial ELISA kit (Bioreba, Switzerland). Further investigation was done using Diba's test. Healthy and infected plants were extracted in extraction buffer. Then 4 microliters of each was poured onto a nitrocellulose membrane, the ELISA plate was blocked with skim milk. The target protein was

detected with an antibody labeled with alkaline phosphatase, with a dilution of 1:500. The bound antibody was revealed by adding NBT/BCIP substrate.

Figure 1: Symptoms of citrus tristeza virus infection in the collected samples

3. Results

3.1.Recombinant coat protein production

To produce sufficient protein for rabbit immunogenicity and further analysis, the major coat protein (CP25kDa) of Tristera virus was selected for expression in bacteria. For this purpose, the expression construct containing the gene encoding coat protein pET28a-CP was expressed in BL21 (DE3) E. coli strain. The results of purification and SDS-PAGE analysis confirmed the high purity and correctness of the protein and the purified protein had the expected size of about 29 kDa (Figure 2). The total amount of purified protein in the culture medium varied from 8 to 20 mg/liter.

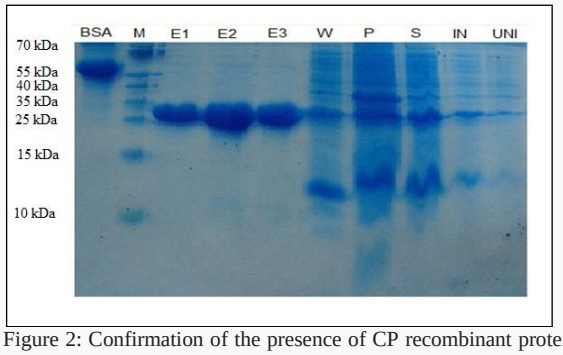

Figure 2: Confirmation of the presence of CP recombinant protein on acrylamide gel. BSA: Bovin Serom Albumin M: protein marker Prestained Protein Ladder SM0671, (Fermentas, Lithuania) E1-E3: protein washing steps from the column W: washing P: precipitation S: supernatant IN: induction UNI: before induction

In order to confirm the detection of CP recombinant protein attached to 6His-tag, the primary antibody Abcam, UK (anti His-tag) and then the secondary antibody GARAP (Abcam, UK) were used in western blot analysis, which shows the successful expression of CP in the cell. It was E. coli. In addition to tracking the expression of CP protein in the bacterial system using monoclonal antibody anti 6xHis-tag in western blot, the efficiency and specificity of polyclonal antibody anti AP was also tested. Western was analyzed (Figure 3).

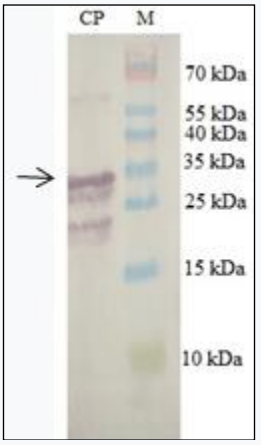

Figure 3: Western blot analysis of purified CP using anti-CPAP polyclonal antibody with a dilution of 1:500, M: Prestained Protein Ladder protein marker SM0671, (Fermentas, Lithuania)., CP: Recombinant coat protein

3.2.Antibody production and identification

The produced coat protein was injected intramuscularly into the rabbit. After each injection, the antibody titer was determined. After 6 weeks, when the antibody titer exceeded 1:65,000, blood was drawn and the serum was separated from other blood cells. The desired antibodies were isolated by the antibody purification kit of Serotec, UK, AbD The results of electrophoresis of the purified antibody indicate the correctness of the purification and two bands were observed, one in the 25 kDa region corresponding to the light strand and the other in the 50 kDa region corresponding to the heavy strand. The concentration of the purified antibody compared to the standard protein (BSA) on polyacrylamide SDS-PAGE gel was estimated to be approximately 1 mg/ml (Figure 4).

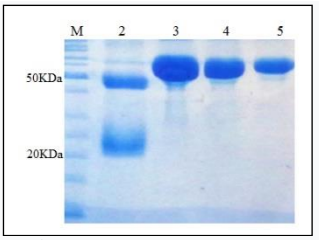

Figure 4- IgG purified by protein column A, M: protein marker (SM0661) Fermentas company, 2: purified antiserum including two bands of 50 kDa (heavy strand) and 25 kDa (light strand) 3-5: standard protein BSA in concentrations of 3.5, 1.75 and 1 microgram

3.3.Detection of infected samples by serological methods

ELISA test was performed to achieve an effective and accurate method to identify plants infected with citrus tristeza by prepared polyclonal antibody. The specificity of the polyclonal antibody prepared against the Tristeza disease agent with the extract of citrus Tristeza infected plants was investigated in the DAS-ELISA test. After taking samples from citrus growing areas in the north and south of Iran, 51 plants suspected of being infected with the Tristeza virus, including leaves, stems and petioles, were selected. To prove the contamination of the samples, the Tristeza virus commercial kit (Bioreba, Switzerland) was used and the sample contamination were confirmed, then, the plant samples were evaluated using the produced antibody. The results of the ELISA test showed that eight samples (H, 9C, 19D, 15D, 13D, 11D, 8D, 3D, 1D) including northern citrus plants and southern citrus plants showed a high infection with the virus, the results indicated a positive antibody reaction. The antibody prepared with the virus was found in the infected samples, the samples of infected citrus fruits in southern Iran were also identified with high specificity by the produced antibody, the produced antibody has a suitable efficiency for detection and separation. Infected samples have a dilution of 1:500 from healthy samples (Figure 5).

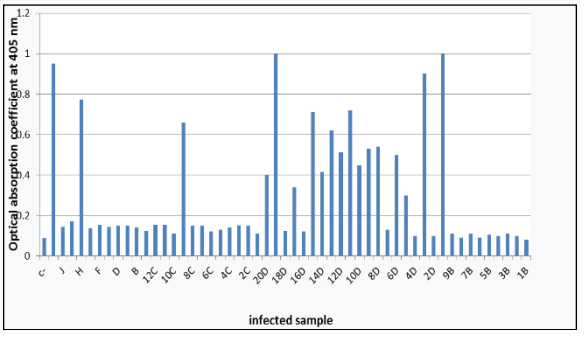

Figure 5- Comparison of plant samples suspected of citrus tristeza by DAS-ELISA test method, with antibody prepared against recombinant protein with a concentration of 1:500

To further evaluate the specificity of the prepared polyclonal antibody, additional DIBA analysis was performed using the extract of the infected citrus plant. The results showed the ability of polyclonal antibody to bind to the CP present in the infected extract and recombinant CP and to identify infected samples, which due to the easy and fast application of this method in examining a large number of samples, it can be used to identify infected gardens on the surface used widely and with many samples (Figure 6, Figure 7 and Figure 8).

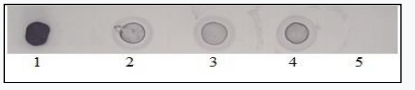

Figure 6- Citrus triestza virus detection in infected samples using polyclonal antiCP antibody bound to alkaline phosphatase with a dilution of 1:500 in Diba test, 1: purified CP protein, 2-4: extract of infected citrus plants, 5: extract healthy plant

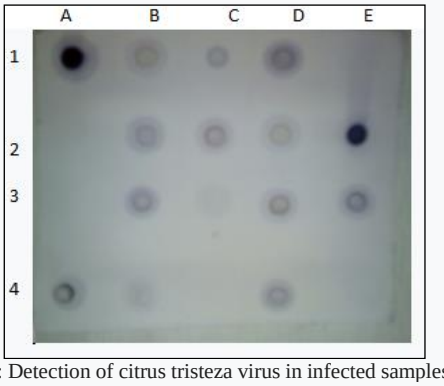

Figure 7: Detection of citrus tristeza virus in infected samples using polyclonal antiCP antibody bound to alkaline phosphatase with a dilution of 1:500 in Diba test, A1, E2: Purified CP protein B1, C1, D1, B2, C2. D2, B3, D3, E3, A4, D4 extract of infected citrus plants, A2: buffer E1, E4: extract of healthy plant

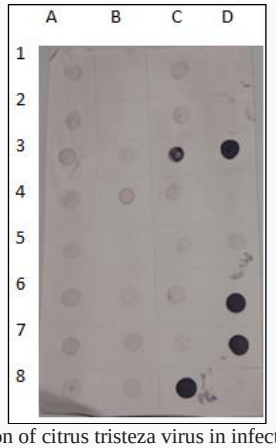

Figure 8: Detection of citrus tristeza virus in infected samples using polyclonal antiCP antibody bound to alkaline phosphatase with a dilution of 1:500 in Diba test, D6, D7, C8, D3: Purified CP protein,A1,A2,A3,A4,A5,A6,A7,A8,B3,B4,B6,B7,B8,C1,C2,C3,C4,C5,C6,C7,D4,D5: extract of infected citrus plants, B1 : buffer D1, D2 : healthy plant extract.

4. Discussion

Tristeza is an important citrus disease worldwide. The most important way to deal with this disease is to use virus-free plants and to prevent the disease by applying quarantine and eradicating the infected plant. For disease control, access to rapid diagnostic methods to identify a wide range of viral strains is a major issue. In this article, using a specific antibody made against citrus tristeza virus, infected samples collected from citrus orchards in the north and south of Iran were identified, which are usually believed to have different pathogenic behaviors. The production of specific antibodies against plant viruses is very complicated due to difficulties in obtaining pure material for immunization. In this direction and for the efficient and simple detection of infected plants, the production of specific antibodies against the virus has been done using recombinant coat protein. The use of recombinant protein as an immunogen to produce antibodies, especially for viruses that are difficult to purify, is of great value, which has solved the problems of virus purification. (Petrovic *et a*l., 2003; Gulati-sakhujaa *et al*., 2009). The purified protein obtained was used to immunize rabbits and prepare a polyclonal antibody against Tristeza disease. Both polyclonal and monoclonal antibodies against Tristeza virus have been produced to detect infected plants (Bar-Joseph 1979. Iracheta, 2005). The produced conjugates identified the pathogen well and showed high specificity. Antibodies produced against virus envelope protein have been proven to be a very powerful tool for pathogen detection. DAS-ELISA and DIBA serological methods have been used for simple and effective detection of plants infected with citrus tristeza virus, which are conventionally used to detect plant pathogens. In addition, serological ELISA methods also have the ability to quantitatively evaluate protein. And the use of this method does not require advanced laboratory equipment, also recombinant proteins are expressed in bacteria and are produced in bulk and cheaply (Kingsnorth *et al*., 2003; Mutasa-Gottgens, 2000). Although the DIBA test is less sensitive compared to DAS-ELISA, the need for a smaller amount of antigen and the quick and easy tracking of a large number of plant samples in field conditions are the advantages of this method (Thomas and Balasundaran, 2001). The sensitivity and specificity of serological reactions mainly depend on the purity of the immunogen. Because viruses cannot be cultured on artificial media, it is recommended to produce recombinant proteins that are very pure immunogens and produce high quality antiserum after injection into rabbits. Citrus tristeza disease is one of the most important diseases of citrus fruits that is widespread in the northern and southern regions of Iran and causes great damage to the product. The methods of combating the vector and preventing the spread of infected plant material and checking resistant plants require the presence of sensitive and specific tracking tools. and serological methods such as ELISA are very powerful tools for tracking and determining plant pathogens (Dewey, 1992; Kingsnorth *et al.,* 2003). Considering that the ELISA kit was made based on the viral sequence of the isolates from the north of Iran, but it also identified the infected samples from the south of Iran well, the use of the antibody produced to identify the infected plants collected from the north and south of Iran shows gave that it can be effectively used for different isolates of Triestzai virus released in different regions of the country.

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