Characterization of *cucurbit yellow stunting disorder virus* and development of polyclonal antibodies using recombinant coat protein

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ABSTRACT

Cucurbit yellow stunting disorder virus (CYSDV) was isolated from cucurbit plants growing in Eastern Province area in Saudi Arabia and characterized using reverse transcription-polymerase chain reaction (RT-PCR). The nucleotide sequence for the coat protein (CP) gene was carried out and submitted in GenBank under accession number JN083790. The phylogenetic tree showed that there are two big clusters and the identity between them 90%. The isolated CYSDV in this study is located in the second cluster with the isolates from Sudan, Iran and the other isolate from Saudi Arabia. The analysis showed that the highest nucleotide identities were 100% with other isolates that isolated from Saudi Arabia and was 98% with other isolates from Iran and Sudan. While the identity was 90% with all other members in the first cluster. The data refers to the isolated CYSDV virus in this study is more related to the isolates from Iran and Sudan. CP gene of CYSDV (CYSDV-CP) was cloned into the expression vector upon induction; the viral protein was expressed as 6XHis-tagged CYSDV fusion protein in *Escherichia coli* cells BL21 strain. The purified protein was immunized by injecting to New Zealand white rabbit using 6 injections at weekly intervals intravenously and subcutaneous. The rabbit blood was collected by bleeding and the antiserum was obtained. IgG was purified and conjugated to alkaline phosphatase. The conjugated antiserum was evaluated by indirect enzyme-linked immunosorbent assay (ELISA). The conjugated antisera that prepared in this study were succeeded to detect the virus in infected plants.

Key words: CYSDV, RT-PCR, phylogenetic tree, 6XHis-tagged fusion protein, indirect ELISA.

INTRODUCTION

Cucurbit crops are widely grown in the Middle East (total 226,492 Ha), and are consumed in large quantities in the traditional diet. In Saudi Arabia, cucurbit crops are grown throughout the year, the total cultivated area is 37,694 Ha and total production is 718,124 tons (Agricultural statistics, Ministry of Agriculture, Saudi Arabia, 2009). Viruses are one of the most limiting factors to the production of cucurbit crops in the Middle East and Saudi Arabia, owing to the widespread distribution of the whitefly vector in all vegetable producing areas of the country (Gill and Brown, 2010). The incidence of whitefly transmitted viruses, at most criniviruses and geminiviruses, has increased enormously during the last three decades due to the fast extension of whitefly populations related to many environmental and man-made factors, such as climatic change (Navas-Castillo *et al*., 2011).

CYSDV is one of the most widespread and damaging viruses in the Middle East which is transmitted by whitefly (Abou-Jawdah *et al*., 2000). CYSDV is transmitted in a semi-persistent, non-circulative manner by whiteflies (Duffus, 1995), commonly by *Bemisia tabaci* biotype B and biotype Q (Berdiales *et al*., 1999). CYSDV can persist for 9 days with half life time of 72.2 h in its vector which is considered as the largest retention time of whitefly transmitted *Closteroviruses* (Sinclair *et al*., 2002). CYSDV was known for a long time to be restricted to the members of *Cucurbitacea* family (Abou-Jawdah *et al*., 2008). Nowadays CYSDV was found in seven distinct members of taxonomic families (El Rahmany *et al*., 2014). Controlling this virus can succeed

only by production of tolerant or resistant plants for this virus and/or the early detection of the virus on the plants.

Recombinant DNA technology may help circumvent purification problems encountered with several viruses (Ling *et al*., 2000; Hourani and Abou-Jawdah, 2003). Expression of different viral genes such as those coding for CP, non-structural proteins, antisense RNAs and ribozymes, among other variants, has resulted in significant protection against infection by the corresponding viruses (Doreste *et al*., 2002). Expression of CP genes in bacteria or in plants can give rise to virus-like particles (VLP), as shown for viruses as different as alfalfa mosaic virus (Yusibov *et al*., 1996), cowpea mosaic virus (Wellink *et al*., 1996). Recombinant virus coat proteins expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses (El-Attar *et al*., 2010). Coat protein gene expression strategy followed by immunization of animals (rabbit) with the fusion protein has many advantages over the conventional immunization. The most important advantage is the elimination of the time consuming and technically demanding steps of protein isolation and purification from plants (Soliman *et al*., 2006). The purification of protein can result in changes in protein confirmation and the loss of epitopes. This problem is probably not encountered during *in vivo* expression of the antigen after coat protein gene expression (Hinrichs *et al*., 1997). In addition, this method causes less distress for the animal; because the administration of the purified protein does not induce any local inflammations (Davis *et al*., 1996; Abou-Jawdah *et al*., 2004). The overall objectives are; characterization of the sequence of the CYSDV-CP gene of the Saudi isolate, expression of the CYSDV-CP gene in *E. coli*, and production of polyclonal antiserum against the expressed protein for the detection of the CYSDV in infected plants.

MATERIALS & METHODS

The work was done at Pest and Plant Diseases Unit (PPDU), College of Agriculture & Food Sciences, King Faisal University, Saudi Arabia.

Source of isolates

Survey was done for incidence and severity of whitefly-transmitted CYSDV. Samples were collected from different parts in Al-Ahsa and Al-Qatif, KSA (Table 1).

Table (1): The number and the type of the collected cucurbit samples from different locations.

Location	No. of Samples	Type of Samples
Al-Hufof, open field and greenhouses	86	Cucumber
Al-Hufof, open field and greenhouses	120	20 Cucumbers - 100 Squashes
Al-Qatif, (Sehat, Al-Malaha)	109	25 Melons - 84 Squashes
Al-Hufof, open field	142	Squash

ELISA

ELISA tests for the detection of CYSDV in the collected cucurbit samples were carried out following the method described by ELISA kits (Bioreba AG, USA).

Extraction of total RNA from plant tissues

Total RNA was isolated from the infected potato plants using RNeasy® Plant Mini Kit obtained from QIAGEN according to manufacturer's instructions.

Design and synthesis of oligonucleotide primers

A pair of specific oligodeoxy nucleotide primers for CYSDV-CP gene were designed (by authors), synthesized and used to amplify the coat protein gene of CYSDV. The forward primer (CYSDV-CP1) sequence: 5`AATAGAATTCATGGCGAGTTCGAGT GAGAAT3`with *Eco*RI restriction enzyme site at the 5` end, and the complementary primer (CYSDV-CP2) sequence was: 5`AAA ACCATGGTCAATTACCACAGCCACCT GG3` with *Nco*I restriction enzyme site at the 5` end. The amplified PCR of the coat protein gene using these primers was generated *Eco*R*I* and *NcoI* compatible ends (5` and 3`, respectively).

One step RT-PCR

One step RT-PCR was done using "Platinum Quantitative RT-PCR Thermo Script One Step System" (Invitrogen, USA) on the samples which gave positive reaction in ELISA test. Total RNA extracted from infected potato plants using RNeasy® plant Mini Kit (Qiagen, USA) were used as templates for one-tube RT-PCR amplification reactions. RT-PCR mixture prepared by combining 12.5 µL of 2X ThermoScript Reaction Mix, 5 µL of total RNA, 1 µL of 10 µM of each primer (forward and reverse primers; Table 1), 0.5 µL of ThermoScript *Taq* Enzyme Mix and the reaction was completed to 25 µL with double distilled water. Reverse transcription reaction started with incubation at 50°C for 30 min, followed by denaturation at 95°C for 5 min. PCR amplification was performed by 35 cycles in a thermal cycler starting with denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min with final extension at 72°C for 10 min. Five microliters aliquots of RT-PCR products were analyzed on 1% agarose gels in 0.5X TBE buffer.

Molecular cloning and nucleotide sequencing of CYSDV-CP gene

The RT-PCR product was ligated directly into pBAD-TOPO® vector (pBAD-TOPO® TA Expression Kit) obtained from Invitrogen, Carlsbad, CA. and the recombinant plasmids were introduced into *E. coli* strain BL21 as described by Sambrook *et al*. (1989). Recombinant plasmids were extracted from selected white colonies using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) digested with *NcoI* and fractionated on agarose gels using 1% agarose gel in 0.5X TBE buffer. The nucleotide sequence of clones having expected inserts were selected for dideoxy sequencing with ABI 377XL automated DNA sequencing instrument and a 5% Long Ranger (FMC) acrylamide gel. Data were analyzed using ABI™ version 3.0 of Sequencing Analysis. All sequencing procedures were completed in Macrogen Company (South Korea). The nucleotide sequences of the coat protein genes of *Cucurbit yellow stunting disorder virus* (CYSDV) were compared and analyzed using DNAMAN Sequence Analysis Software (Lynnon BioSoft. Quebec, Canada) with those of other isolates available in GenBank.

Expression of the CYSDV-CP gene in *E. coli*:

To express CYSDV-CP gene in *E. coli* BL21, liquid LB medium containing 60 µg/ml ampicillin was inoculated with a single recombinant *E. coli* colony and incubated overnight at 37ºC with shaking (250 rpm) to obtain a saturated culture. 50 ml of LB medium containing ampicillin was inoculated with 1 ml of saturated culture and incubated at 37ºC with vigorous shaking to reach an OD_{600} = ~ 0.5. One ml of the uninduced culture was centrifuged at 12,000 rpm for 1 min at room temperature and the cell pellet was stored at –20ºC. The remaining culture was induced by adding the

optimal amount of the inducer (L-arabinose). To optimize the inducer concentration and the time of induction; 5 different concentrations of L-arabinose were used at final concentrations of 0.00002 %, 0.0002 %, 0.002 %, 0.02 % and 0.2 %. 1 ml aliquots of the induced cultures were removed at 2, 3, and 4 h after induction and immediately centrifuged at 12,000 rpm for 1 min at room temperature and the cell pellets were stored at –20ºC. The remaining induced cultures were centrifuged at 4,000 rpm for 10 min at 4ºC and the bacterial pellets were stored at –80ºC for further use. The pellets (from –20ºC) were resuspended in 100 µl of 1X SDS gel loading buffer, (50 mM Tris-Base, pH 6.8, 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol) and heated to 100ºC for 3 min, and then centrifuged at 10,000 rpm for 1 min at room temperature. Then the expressed protein was separated from other bacterial proteins on a 12% SDS-PAGE (Sambrook *et al*., 1989).

Purification of 6XHis-tagged CYSDV fusion protein:

The rapid purification of 6XHis-tagged CYSDV fusion proteins from bacteria (the pellets from –80ºC) was done using the B-PER^{TM} 6XHis Spin Purification Kit (Pierce, Cat. No. 78300) as follows: The pellets were thawed on ice before starting protein extraction. The cell pellet was resuspended in 10 ml B-PER reagent by pipetting up and down until the cell suspension was homogeneous. Once a homogenous mixture was established, shaking gently at room temperature for 10 minutes was done. Soluble proteins were separated from insoluble ones by centrifugation at 14,000 rpm for 15 min. The supernatant (soluble fraction) was transferred to a 15 ml capped conical centrifuge tube and 1 ml the nickel-chelated agarose was added to the soluble fraction, then shaking for 10 min at room temperature and spinning at 2,500 rpm. Supernatant was removed and the resin was resuspended with 0.25 ml of wash buffer. (The total volume was about 0.75 ml). The resin-bound, 6XHis fusion protein was transferred to the B-PER spin column and centrifuged at 10,000 rpm for 2 min then 0.5 ml of wash buffer was added to the B-PER spin column, incubated for 5 min and centrifuged at 10,000 rpm for 2 min. The 6XHis fusion protein was eluted by adding 0.5 ml of the elution buffer, incubated for 5 min and centrifuged at 10,000 rpm for 2 min. The last step was repeated 3 times for a total 4 fractions (each fraction was collected into a separate collection tube) and the eluted 6XHis fusion protein assayed by 12% SDS-PAGE.

Immunization of rabbit:

One New Zealand white rabbit, weight ~ 4 kg, was used to develop antisera raised against the expressed CYSDV-CP. The immunization of rabbit was done using 6 injections at weekly intervals; the first and the fifth ones were intravenous each with 750 µl of purified protein (1 mg/ml) while the rest of injections were subcutaneous each with 1 ml of purified protein (1 mg/ml) emulsified with an equal volume of Freund's complete adjuvant (Sigma, Cat. No. F-5881) in the second injection and Freund's incomplete adjuvant (Sigma, Cat. No. F-5506) in the rest of subcutaneous injections (Table 2).

Blood collection and serum processing:

Bleeding the rabbit was carried out two weeks after the last injection. Blood was obtained from the rabbit by veinal pathway through the marginal ear vein. Once the antiserum reacted positively with CYSDV infected tissues in ELISA test, the whole blood was collected. The blood was kept for one hour at room temperature for clotting then the clot was released and the blood was heated at 37°C for 30 min then stored at 4°C for overnight. The serum was decanted from

the clot and centrifuged at 2,000 rpm to remove cell debris. The serum was filtered through a 0.2 µm millipore filter (Corning, Cat. No. 21052-25), and brought to 0.025 % sodium azide. Aliquots of serum, mixed with equal volumes of glycerol, were stored at –20°C.

Purification of immunoglobulin G (IgG):

The IgG fractions (IgG from the expressed CYSDV-CP) were partially purified by selective precipitation with high concentrations of salts (ammonium sulfate, final concentration 40 %, pH 7.0) and dialysis three times against 500 ml of the 1X PBS (phosphate buffered saline) [10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.1 mM Na_2EDTA, pH 6.8] (Ball *et al*., 1993).

Table (2): Rabbit injection schedule.

Type of injection	Volume (ml) of purified protein	Volume (ml) of adjuvant	Type of adjuvant
intravenous	0.75	-	
subcutaneous	1	1	complete
subcutaneous	1	1	incomplete
subcutaneous	1	1	incomplete
intravenous	0.75	-	
subcutaneous (poster injection)	1	1	incomplete

Indirect ELISA:

One hundred mg of leaf tissues were homogenized in 2X PBS. Plates were coated with plant extracts (200 µl/well) and incubated overnight at 4°C, then blocked with 200 µl of blocking buffer (1X PBS, 0.5% of BSA) for 1 hr at room temperature. 200 µl of the anti-PVX was added to each well then incubated at 37°C for 3 h. 200 µl of the diluted secondary antibody alkaline phosphatase conjugated (Anti-Rabbit antibody) were added to each well and incubated for 1 h at 37°C. All washing steps between incubations were performed with 1X PBS-T buffer. Freshly prepared pNPP substrate was added to the wells. The plate was incubated for 30 min at room temperature away of direct light. The reaction was stopped by addition of 50 µl of 3M H_2SO_4 to each well and the absorbance was measured at 405 nm.

RESULTS

Survey of major cucurbit growing areas in Saudi Arabia:

The collected cucurbits plants from some different locations showed typical CYSDV symptoms such as yellowing, stunting and malformations (Fig. 1).

Amplification of the CYSDV-CP gene:

RT-PCR amplification of viral RNAs were carried out on the total RNAs isolated from infected plants using specific primers designed to amplify the coat protein genes. Electrophoresis analysis of RT-PCR products showed single amplified fragments of 756 bp for CYSDV-CP respectively. No fragments were amplified from the RNAs extracted from symptomless or healthy plants (Fig. 2). The expected size was obtained at 756 bp.

Fig. 1: Field survey and collecting of cucurbit samples. a: mosaic and yellowing on squash leaf, b and c: stunting and malformations on leaves of squash plants, d: general view of squash field.

Molecular cloning and nucleotide sequencing of CYSDV-CP gene:

RT-PCR products obtained with the infected samples were cloned and sequenced to identify the suspected CYSDV strain. The core region of the CP, which was amplified with the primers CYSDV-CP1 and CYSDV-CP2 was sequenced and submitted in GenBank under accession numbers JN083790. Phylogenetic analysis was carried out for the partial sequences (CP core region) of the obtained sequences for Saudi Arabian isolates and representative sequences of CYSDV isolates from Iran, Saudi Arabia, Egypt, Sudan, USA, Guatemala, Jordan and Cameron that available in GenBank. The phylogenetic tree in (Fig. 3) showed that there are two big clusters and the identity between them 90%. The first cluster includes isolates from Egypt, USA, Spain, Guatemala, Jordan and Cameron and the second cluster includes isolates from Saudi Arabia, Iran and Sudan and the isolated CYSDV in this study is located in the second cluster with the isolates from Sudan Iran and the other isolate from Saudi Arabia.

Expression of the CYSDV-CP gene in *E. coli:*

Expression of CYSDV-CP was induced in *E. coli* strain BL21 transformed with the recombinant plasmid by addition of L-arabinose. CYSDV-CP was expressed as a fusion protein with an estimated molecular weight of about 30.5 KD, which contains about 2 KD of tagged fragment from the vector and 28.5 KD from the CYSDV-CP. The expression of the protein increased continuously over time and reached maximum at 3 h after induction at 37°C. The optimal concentration of the inducer (L-arabinose) is 0.00002 % (Fig. 4). Since the expression of the protein was specifically induced and its size corresponded to the expected size, it was concluded that this protein was the CYSDV-CP (Fig. 5).

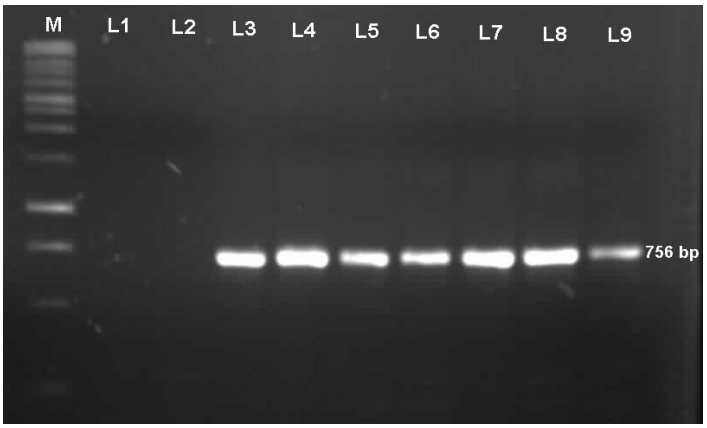

Fig. 2: Gel electrophoresis for RT-PCR product using the degenerate primers CYSDV-CP1 and CYSDV-CP2. M: 1 Kb DNA ladder; lanes 1, 2: healthy plant as negative control, lanes from 3 to 9: infected cucurbit plants with CYSDV.

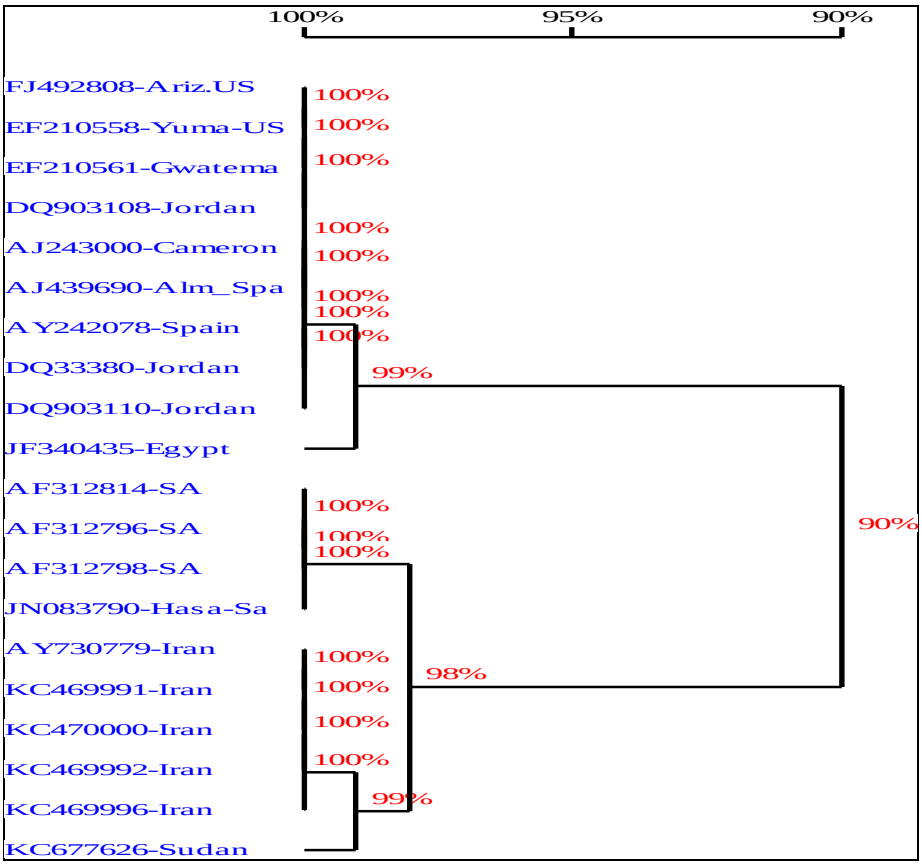

Fig 3: Neighbor joining tree showing the genetic distance among the core region of the coat protein gene of CYSDV genomes. The sequences were either obtained in this study or selected from GenBank and were aligned with the optimal alignment method of DNAMAN The tree was set up with a Jukes and Cantor distance matrix using the Neighbor Joining method of DNAMAN.

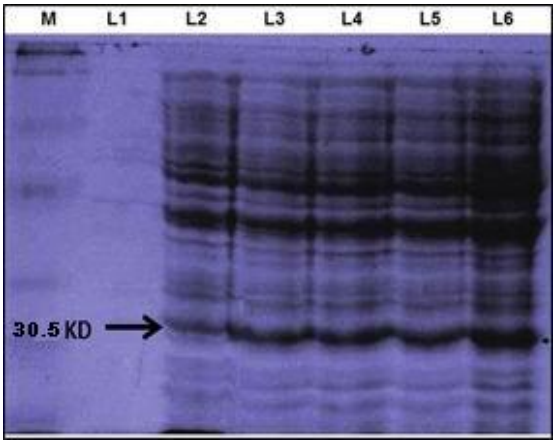

Fig. 4: SDS-PAGE for induced and uninduced bacterial cells expressing CYSDV-CP. M: the blue ranger prestained protein marker (PIERCE), L2, L3, L4, L5, and L6: induced cultures of the same clone at five different concentrations of the inducer (L-Arabinose); L6: is the uninduced culture of the same clone. L-Arabinose final concentrations are: 0.00002% in L6, 0.0002% in L5, 0.002% in L4, 0.02% in L3, and 0.2% in L1.
Expected size was ~ 30.5 KD.

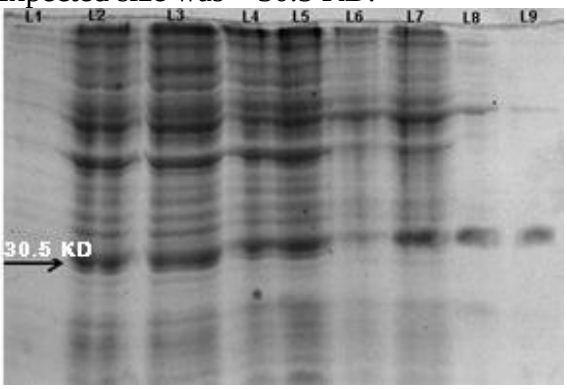

Fig. 5: SDS-PAGE for bacterial cells expressing CYSDV-CP and the purified CYSDV-CP. L1: uninduced culture; L2 and L3: induced cultures; L4: bacterial cell lysate; L5: pellet collected after chemical lysis of the induced culture (insoluble proteins); L6: supernatant collected after resin purification; L7: purified protein (1st elution); L8: purified protein (2nd elution); L9: purified protein (3rd elution).

Confirmation of the produced antibodies using indirect-ELISA:

Determination of the working dilutions of IgG raised against CYSDV-CP is shown in Table 3. Wells in which colour develops indicate positive results while that in which there is no significant colour development indicates negative results. The results of the ELISA revealed that IgG is able to recognize CYSDV from infected tissues at dilution of 1:2000. The ELISA tests were done on 457 samples collected from Al-Ahsa and Al-Qatif (Table 4) using obtained antibodies for CYSDV and antibody obtained from kit (as control). Two independent replicates for each cucurbit sample were made in order to minimize the variations in readings and normalize the data related to healthy leaves (negative control). From the table (Table 4) we can conclude that, 75 samples from total 457 samples were positive with CYSD antibody against this virus with 16.4% of infection.

Table (3): Determination of series of dilutions of IgG against infected and healthy samples by indirect ELISA.

IgG Dilution	Absorbance at 405 nm	
	Infected Sample	Healthy Sample
1/100	1.442	0.053
1/200	1.034	0.061
1/500	0.823	0.057
1/1000	0.596	0.051
1/2000	0.328	0.063
1/4000	0.115	0.065
1/8000	0.076	0.054
1/16000	0.067	0.062
1/32000	0.056	0.049
1/64000	0.052	0.047

Table (4): The result of ELSA test for the detection of CYSDV in the collected samples from the field.

Location	N°. of Samples	Result of ELISA test (N°. of the positive samples for CYSDV)	Percentage of infection (%)
Al-Hufof, open field and greenhouses	86	8	9.3
Al-Hufof, open field and greenhouses	120	21	17.5
Al-Qatif, (Sehat, Al-Malaha)	109	0	0
Al-Hufof, open field	142	46	32.4
Total	457	75	16.4

DISCUSSION

The fast spread of CYSDV in the Mediterranean Basin and in North America makes it one of the most typical examples of emerging viruses infecting cucurbits (Brown *et al.*, 2007). Survey was done for incidence and severity of CYSDV. 457 Samples were collected from different parts in Al-Ahasa and Al-Qatif, KSA. The collected cucurbit samples showed typical CYSDV symptoms such as yellowing, stunting and malformations. In order to detect CYSDV in symptomatic plants PCR analysis was performed using degenerate primers CYSDV-CP1 and CYSDV-CP2. The expected size at 765 bp was obtained with 125 out of 390 of the collected cucurbit samples with percentage of 32%. PCR analysis was performed using primers CYSDV-CP1 and CYSDV-CP2 to amplify about 750 bp of the full length of coat protein gene of CYSDV from the infected collected plants and Fig. 3 shows the expected size bands of the coat protein gene. pBAD TOPO® TA Expression Kit provides a highly efficient one step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for regulated expression in *E. coli*. Several white colonies resistant to ampicillin were selected to test for recombinant plasmids containing the CYSDV-CP gene. Restriction enzyme digestion with *Nco*I released the cloned gene due to the presence of *Nco*I site at 5` end in the pBAD-TOPO vector and the other NcoI site is located in the complementary primer. The phylogenetic tree in (Fig. 3) showed that there are two big clusters and the identity between them 90%. The first cluster includes isolates from Egypt, USA, Spain, Guatemala, Jordan and Cameron and the second cluster includes isolates from Saudi Arabia, Iran and Sudan and the isolated CYSDV in this study is located in the second cluster with the isolates from Sudan Iran and the other isolate from Saudi Arabia. The analysis showed that the highest nucleotide identities were 100% with other isolates that isolated from Saudi Arabia by Rubio *et al*, (2001) and was 98% with other isolates from Iran and Sudan. While the identity was 90% with all other members in the first cluster. The data refers to the isolated CYSDV virus in this study is more related to the isolates from Iran and Sudan those are very close to the Saudi Arabia and the whiteflies which is the transmitted vector of this virus may it plays the main roll to transmit the virus from Iran to Al-Ahsa, Saudi Arabia.

CYSDV-CP was expressed as a fusion protein with an estimated molecular weight of about 35 kDa, which contains about 2 kDa of tagged fragment from the vector and 33 kDa from the CYSDV-CP. Separation and analysis of bacterial proteins by SDS-PAGE revealed a high level of expression of the 35 kDa protein only in the induced bacterial cells containing the recombinant

plasmid. This protein was readily detectable as the most abundant cellular protein. Cell fractionation experiments showed that the majority of the fusion protein was found in soluble form inside the cell and the protein was not expressed in non-induced cells with the same plasmid. Since the expression of the protein was specifically induced and its size corresponded to the expected size, it was concluded that this protein was the CYSDV-CP. The expression of the protein increased continuously over time and reached maximum at 3 h after induction at 37°C. The optimal concentration of the inducer (L-arabinose) is 0.00002%. The purification of the proteins from bacterial cells indicated that the expressed protein was soluble, the first elution contains the highest concentration of the purified protein and used for rabbit immunization while the third elution contains very low concentration of the protein. In western blotting experiment, the fusion protein reacted with rabbit polyclonal antiserum directed against CYSDV. Antisera against plant viruses can be produced in several animal species. Rabbits are more commonly used because they can produce large volumes of serum and they respond well to most plant virus antigens. The interaction between the antigen and the specific antibody is the basis for serological tests. CYSDV-CP of the Saudi isolate was successfully cloned, expressed and purified under non-denaturing conditions. The suitability of produced purified antibodies for use in diagnostic tests was compared to an efficient commercial DAS-ELISA kit. This study addressed the possibility of using recombinant CYSDV-CP to produce CYSDV specific antisera and to test their suitability for use in serological diagnostic assays for surveys or in certification programs. This investigation suggested that the recombinant virus coat proteins expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses. The indirect ELISA is used primarily to determine the strength and/or amount of antibody response in a sample, whether it is from the serum of an immunized animal or the cell supernatant from growing hybridoma clones (Salazar, 1996). Readings from the CYSDV-infected leaves were compared to those of healthy leaves. Readings from healthy extracts were relatively high and attempts to reduce the back ground level by treatment of samples with DIECA were not successful. Three independent replicates for each cucurbit sample were made in order to minimize the variations in readings and normalize the data related to healthy leaves (negative control). Determination of the working dilutions of IgG rose against the expressed CP through indirect ELISA is shown in table 3. Wells in which color develops indicate positive results while that in which there is no significant color development indicates negative results. The results of the indirect ELISA revealed that the raised IgG are able to recognize CYSDV from infected tissues at dilutions of 1:2000.

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