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RESEARCH ARTICLE

Antiserum production, biological and serological detection of *Cucurbit yellow stunting disorder crinivirus* (CYSDV) in Egypt.

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Abstract

Cucurbit yellow stunting disorder crinivirus (CYSDV) causes significant yield losses in the field- and greenhouse grown cucurbits. In Egypt, it has become serious problems for open fields and greenhouses production of all cucurbit (Family: *Cucurbitaceae*) plants *i.e.*, cantaloupe, cucumber, pumpkin, squash and watermelon. Inspection of (*Cucumis sativus* L.) cv. El-Safa plants grown in the fields exhibited extensive interveinal yellowing and green veins are most apparent on the older leaves and reduced plant vigor, whereas new growth appears normal. Whilst in the greenhouse trail, CYSDV-infected plants showed prominent interveinal yellowing typical to naturally infected ones, 3-4 weeks after whiteflies inoculation. In whiteflies transmission experiments, the maximum transmission efficiency (86%) was recorded, when applying fifteen insects in each treatment to transmit CYSDV after 48hr for both AAP and IAP. The highest percentages reached to 93 and 86% respectively, in CYSDV-naturally infected and whiteflies inoculated squash cv. Escandarani plants. The lowest percentages were recorded in watermelon and pumpkin being 6% and 13% respectively, in natural infection and whiteflies transmission. In Egypt, CYSDV infects noncucurbit plants belonging to the families *Amaranthaceae, Chenopodiaceae, Leguminosae (Fabaceae), Malvaceae and Solanaceae.*
The purified virus has A_{max} at 260 and A_{min} at 240 of 1.3. Virus yield was 20mg/200g of fresh weight. Evalution of cucumber varietal reaction against CYSDV-infection using the induced antibody (CYSDV-Pab) in DAS-ELISA, after 1h and overnight incubation with paranitrophenyl phosphate (PNPP) substrate gave nearly similar results. The induced polyclonal antibody (CYSDV-Pab) was also evaluated in detecting CYSDV antigen in the infected plants through serological tests *i.e.*, tissue and dot blot immuno-printing assay (DBIA&TBIA, respectively) in naturally-and whiteflies infected plants. Both DBIA and TBIA proved to be effective in differentiating between healthy and infected samples, faster, sensitive and reliable techniques for the detection CYSDV in cucumber leaf petioles.

Introduction:-

Cucurbit plants are infected by many whitefly-transmitted viruses in the genera *Criniviruses* which are emerging and considered as an obstacle and cause severe economic losses in many crops production worldwide. *Cucurbit yellow stunting disorder virus* (CYSDV) is one of the most destructive pathogen affecting-fields and greenhouse-grown cucurbit plants in the tropical and subtropical regions worldwide (Tzanetakis *et al.*, 2013; Orfanidou *et al.*, 2014; EPPO, 2014; Amer, 2015 and Wintermantel *et al.*, 2016). Typically, yellowing disease caused by CYSDV-infection is associated with a loss of photosynthetic capability, often characterized by interveinal yellowing or reddening of leaves, chlorotic mottle, leaf brittleness, reduced plant vigor, yield reductions, and early senescence, depending on

the host plant affected. CYSDV has recently become a significant production threat throughout cucurbit production regions in the Middle East, the Mediterranean Basin (Lecoq and Desbiez, 2012), North Africa and Southern Europe as well as the Canary Islands, the Southern United States, Mexico, and Central America (Bananej *et al*., 2013). *Cucurbit yellow stunting disorder virus* (CYSDV) is a member of the genus *Crinivirus* within the family *Closteroviridae* (Wintermantel *et al*., 2009; Fidan *et al*., 2012; and Abrahamian *et al*., 2015). It has a bipartite single stranded plus-sense RNA encapsidated in long and flexuous particles. CYSDV has emerged as a serious whitefly-transmitted virus of cucurbit crops, causing between 30-100% yield losses (Aguilar *et al*., 2006 and Abrahamian and Abou-Jawdah, 2014). CYSDV was initially discovered in 1982 in the United Arab Emirates (Hassan and Duffus, 1991). Since, then CYSDV has progressively spread to many cucurbit production regions in Africa (Desbiez *et al*., 2000 and Yakoubi *et al*., 2007), Asia (Abou-Jawdah, *et al*., 2000; Liu *et al*., 2010; McCreight and Wintermantel, 2011; Lecoq & Desbiez, 2012 and Keshavarz *et al*., 2013), Europe (Celix *et al*.,1996; Desbiez *et al*., 2001; Papayiannis, *et al*., 2011) and in USA (Brown *et al*., 2007; Wintermantel, *et al*., 2009; Tzanetakis *et al*., 2013). Nowadays, CYSDV has become a significant production threat throughout cucurbit production regions in the Middle East, the Mediterranean Basin (Lecoq and Desbiez, 2012), North Africa, and Southern Europe as well as the Canary Islands, the Southern USA, Mexico, and Central America (Bananej *et al*., 2013).

For this reason, the present investigation greatly expands to: (i) identify CYSDV bilogically *i.e*., particles examination by electron microscope, whiteflies transmission and varietal reaction, (ii) purify CYSDV and production of CYSDV-antiserum (Polyclonal antibody, CYSDV-Pab) and (iii) evaluation of the induced CYSDV-Pab in detecting CYSDV-antigen by various techniques *i.e.*, DAS- and indirect ELISA, tissue and dot blot immuno-printing assays (DBIA & TBIA, respectively).

Materials and Methods:-

1- Virus identification and source of the isolate:-

The CYSDV isolate used in this study was originally obtained from cucumber (*Cucumis sativus* L.) cv. El-Safa plants are heavily infested with whiteflies insect *Bemisia tabaci* Genn., grown under greenhouse conditions and fields at the experimental station of Fac. of Agric., Cairo Univ. The plants were exhibited severe yellowing and green veins on the lower leaves, while juvenile leaves near the apex remain green. The results were confirmed by DAS-ELISA using CYSDV-Pab antiserum. Petioles of detached leaves form infected cucumber, heavily infested with insects were placed in tubes containing water. Then they were transported and kept under cages with healthy cucumber plants to allow feeding of viruliferous insects. Whiteflies-inoculated cucumber plants were reacted with interveinal chlorotic spots followed by yellowing and green veins maintained as a CYSDV stock for further studies in the greenhouse belonged to Plant Pathology Dept.

Transmission studies:-

Mechanical transmission and host range studies:-

To insure that the natural symptoms are mixed (CYSDV & *Cucumber vein yellowing virus* [CVYV]) or single infection, DAS-ELISA positive samples were used for mechanical inoculation on healthy cucumber, squash and melon plants. Sap was obtained by grinding symptomatic leaves in cold phosphate buffer, pH 8.1 containing 0.2M Na_2HPO_3, 0.02M Na-EDTA and 1.5% Triton X-100. Ten plants of each test plants were inoculated. Control plants were inoculated with the same buffer. Inoculated plants were kept under cages covered with fine muslin in an insect-proof greenhouse for a period of one month. Later on, cages were removed and plants were watched for symptom development until fruit set.

Maintenance of virus culture,whiteflies and plants:-

Viruliferous adults of whitefly (*B. tabaci* Genn.) were collected from CYSDV-infected cucumber cv. El-Safa plants. Adult insects were reared on sweet potato plants grown in insect-proof glass cages at 24-27°C for 7 days for ovipositor. The virus was isolated and biologically purified through several passages (6 generations) of whiteflies on as described by Ghanem (2002) and Ghanem *et al.* (2003). For whitefly transmission experiments, virus-free (non-viruliferous) *B. tabaci* colonies were maintained on healthy cucumber plants cv. El-Safa in an insect-proof glasshouse. After the 6th generation, non-viruliferous adults of whiteflies were confined to CYSDV-infected cucumber plants for virus acquisition to be as a source for the virus in further experiments.

Virus-vector relationships:-

Whiteflies transmission trial was done as previously described by Ghanem *et al.* (2003). Tests were performed for determination of the transmission efficiency, after acquisition access period (AAP) and inoculation access (IAP).

Fifteen non-viruliferous adults of whiteflies were confined to CYSDV-infected cucumber plants for 48h AAP. After 48h, insects were transferred to healthy cucumber seedlings for 48hr IAP and kept in glass cage. Control plants were obtained following the same scheme but using non-viruliferous whiteflies. The results were examined visually and calculated as well as confirmed by DAS-ELISA test.

Concerning experimental of reservoir hosts, to determine the infectivity of CYSDV on non-cucurbit plants, large numbers of *B. tabaci* were allowed for acquisition access periods (AAP) of 48h on CYSDV-infected cucumber as a source plants, approximately 2 to 3 weeks old exhibiting symptoms. Following virus acquisition, whiteflies were transferred to cages containing test plants and fifteen whiteflies each were clipped to the under side of a leaf of each test plant for 48h transmission periods. Following inoculation of plants with CYSDV via whiteflies, the plants were sprayed with a pesticide to kill adult and immature whiteflies, and placed in net cages within growth chamber for 4 weeks. Growth chambers were maintained at 27°C for the duration of experiments. One week post-inoculation, the leaves of the inoculated plants were removed to prevent any remaining whitefly nymphs from maturing to adults. In order to determine if newly identified non-cucurbit hosts (See below in Table 2) could serve as reservoirs of CYSDV for back inoculation to cucurbits. CYSDV-infected leaves of newly identified hosts were collected from the field and used as a source of the virus. The results were examined visually and confirmed by transmission electron microscope (TEM).

Virus examination using electron microscopy:-

The carbon-coated grids were floated on sap of CYSDV-infected cucumber leaves after centrifuged10.000rpm/3min for partial clarification, then stained with 2% phosphotungestic acid and air dried. Grids were examined with transmission electron microscope JEOL (JEM-1400 TEM, Japan) at the candidate magnification. Images were captured using CCD camera Model AMT. Virus examination was done in Research Park (FARP), TEM Lab., Fac. of Agric., Cairo University.

Purification of CYSDV and antiserum production (Polyclonal antibody):-

Three weeks after whiteflies inoculation cucumber cv. El-Safa plants were used as a source for virus purification. All steps in purification were carried out at 4°C after initial homogenization of tissues. Concerning production of polyclonal antibody (CYSDV-Pab), a Newzealand white rabbit was given 5 intramuscular injections each with (1ml) of purified virus emulsified with an equal volume of freund's incomplete adjuvant at weekly intervals. One intravenous injection without the adjuvant was given in the 6th week. Antiserum was collected weekly intervals starting 4-weeks after the last injection according to Aly-Manal (1997). The UV-absorption spectrum of the supernatant containing virus isolate was measured to evaluate the purity and concentration using an extension coefficient $A^{0.1}$% 260nm 1cm.

Serological detections:-

Efficiency of the induced polyclonal antibody (CYSDV-Pab) was evaluated in detecting CYSDV-antigen in the infected plant tissues through several serological tests such as indirect- & DAS ELISA methods and Dot blot immuno-printing assay (DBIA) as well as tissue blot immuno-printing assay (TBIA) on nitrocellulose membrane as the following:

Direct- and indirect ELISA assays:-

DAS-ELISA was done to evaluate the CYSDV-Pab in: (i) determination of the optimal concentration of IgG & IgG-AP in detecting CYSDV-infected tissues using two different buffers, (ii) evaluation cucumber varietal reaction against virus infection.

In order to determine the optimal concentration of CYSDV-Pab, IgG and IgG-enzyme (alkaline phosphatase) conjugate (IgG-AP) were prepared and purified according to standard procedures, then ELISA tests were carried out as previously described (Clark & Adams, 1977). In order to perform DAS-ELISA, the purified antibodies (IgG) were used at concentrations of 1:250 and 1:500 in the two citrate buffers (pH 6) or general buffer (pH 7.4) for incubation at 37°C for 3h. Also, alkaline phosphatase-conjugated antibodies were used at concentrations of 1:250, 1:500 and in PBS (pH 7.4) containing Tween 20 (0.05%), polyvinylpyrrolidone (PVP) K 25 (2%) and ovalbumin (0.2%), and incubated at 37°C for 2 or 3h. To determine the optimal concentration of IgG &IgG-AP for detecting CYSDV-infected tissues, various concentrations were used. Leaf extracts from cucumber samples were added to the plates using 100µl/well after diluting the infectious crude sap to 1/10 in the two buffers, then incubated overnight at 4°C. The antigen was detected with enzyme-labeled specific antibody using different dilutions *e.g.*, 1/250 and 1/500

in conjugate buffer (PBS-T containing bovine serum albumin 0.2% [w/v] pH 7.4, then incubated for 3h at 37°C or overnight at 4°C. The plates were washed and dried. Then paranitrophenyl phosphate (PNPP) substrate (1.0mg/ml) dissolved in substrate buffer/pH 9.8 composed of 9.7% diethanolamine and 0.2% NaN_3 was added. After incubation with substrate for 2h, optical density was measured using 405_{nm} with ELISA reader.

Concerning indirect ELISA that described by Aly-Manal (1997), 1g of leaf tissue was homogenized in 10ml of coating buffer (pH 9.6). ELISA plates were coated with 100µl of plant extracts and incubated at 4°C overnight. Cross-absorbed antiserum was then added at a dilution of 1/1000 (100µl/well). The plates were added and incubated at 37°C for 3h before 200µl of 1:1000 dilution, AP-labeled anti-rabbit IgG was applied. After incubation for 2h at 37°C, 200µl of freshly prepared *p*-nitrophenyl phosphate (PNPP, 1.0 mg/ml) in 0.1M diethylamine substrate buffer (pH 9.8) was added to each well. ELISA plates were washed three times with PBS-T after each step. Detection of CYSDV-infection using DAS- and indirect ELISA were done on various cultivars of cucurbit plants *i.e.* cantaloupe, cucumber, pumpkin, squash and watermelon to determine their reaction under natural and greenhouse conditions (See, name of cultivars bellow Table 1). Concerning whiteflies inoculated trail in the greenhouse, fifteen seedlings were used per each treatment. Fifteen viruliferous adult insects/seedlings were kept under cages for 48h IAP. The whiteflies inoculated plants were sprayed with an insecticide to kill insects and kept in an insect-proof greenhouse for a period of one month. Later on, cages were removed and plants were visual inspected for symptoms development until fruit set. Control seedlings were exposed to non-viruliferous insects and treated as the above mentioned. The results were confirmed by indirect and direct ELISA tests. Percentages of CYSDV-infection were calculated according to the following equation:

$$(\%)\ \text{infection} = \frac{\text{Number of infected plants}}{\text{Number of total plants}} \times 100$$

DBIA and TBIA assays:-

Protocols of DBIA and TBIA assays were performed to detect CYSDV-infection using Nitrocellulose membrane (NCM) to confirm ELISA detection. The immuno-blotting procedure was done to determine the distribution of CYSDV-antigen in infected tissue sections (petioles and midrib) as described by Fargette *et al*. (1996) and Ghanem (2002). Preparations were treated with equal volumes of TBST buffer containing 0.01 M Tris-HCl, 0.05% Tween-20 and 0.15 M NaCl, pH 8.0 in TBIA test; chromogenic substances were used for color development. Nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3 indolyl phosphate (BCIP) complex was used for purple-color development in positive reaction compared with green color for negative reaction.

Evaluation of cucumber varietal reaction using the induced antibody:-

This trail was conducted to evaluate the produced antibody (CYSDV-Pab) in detecting cucumber varietal reaction against CYSDV-infection using DAS-ELISA at different incubation periods with PNPP substrate. Twelve cucumber cultivars (*Cucumis sativus* L.) *i.e.*, El Safa, Kuck, Sina 1, El Sham, Hybrid, Ku-12, Beta Alpha America, Hybrid 173, Ku-10, Al Zeam, Monier and Sina 2, were used including traditional cultivars and breeding lines. Symptoms of CYSDV-infection were observed and recorded for one month, and then samples were collected to be tested. Control seedlings were exposed to non-viruliferous insects and treated as the above mentioned. Leaf extracts from cucumber cv. EL Safa showing typical CYSDV-symptoms were used as positive controls. Absorbance readings (optical density, O.D.) were measured at 405_{nm} after four incubation period *i.e.*, 10, 30 min, 1h and overnight.

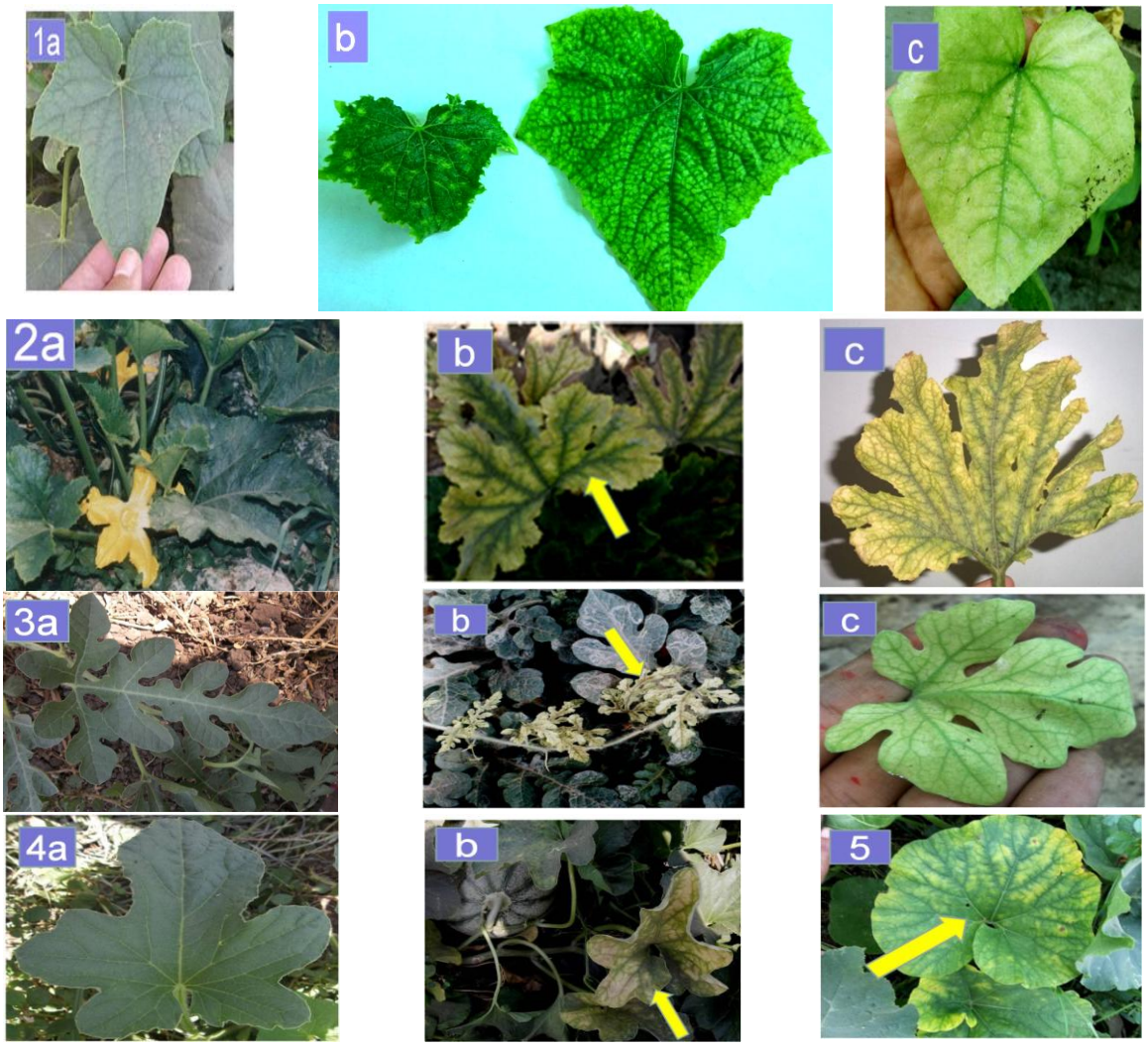
Results and Discussion:-

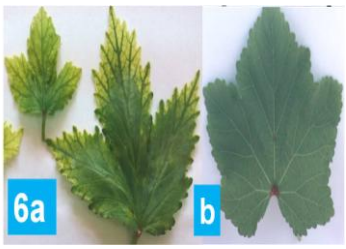
Inspection and symptoms of CYSDV:-

Yellowing diseases of field- and greenhouse grown cucurbits are becoming increasingly important in many areas worldwide and particularly in Egypt. All the causal agents of these diseases belonging genus *Crinivirus*, is one of the three genera in the family *Closteroviridae* which include viruses with segmented genomes, transmitted by whiteflies. Nowadays in Egypt, *Cucurbit yellow stunting disorder virus* (CYSDV), genus *Crinivirus* has been considered as a major threat and devastating for cucurbit crops either grown in the fields or greenhouses. CYSDV often causes symptoms that are readily mistaken for physiological defect and pesticide phytotoxicity.

Many of the synergistic effects have been found in cucurbits plants infected with combinations of criniviruses such CYSDV and viruses belonging to other genera like *Cucumber vein yellowing virus* [CVYV] (Fidan*et al.*, 2012; Gil-

Salas *et al*., 2012 and Abrahamian *et al.*, 2015). CYSDV is not transmitted mechanically, while CVYV is transmitted by same method. To insure that the source plant used is infected only with single virus, the inspection reveals that CYSDV induces interlveinal chlorosis and yellowing symptoms on leaves of cucumber (*Cucumis sativus* L.), melon (*C. melo* L.), squash (*Cucurbita pepo* L.) and watermelon (*Citrullus lanatus* Thunb. "Matsum. & Nakai") are the most prevalent symptoms in all cucurbit plantations. It causes severe symptoms on all cucurbit plants, starting by interveinal chlorotic spots on middle leaves and spreading upward, resulting in yellowing and downward of the entire older leaves except for the veins, which remain green. Juvenile leaves reacted with small interveinal chlorotic spots (Figs. 1-5). Our observations reveal that the yellowing symptoms are accompanied by substantial reductions of fruit yield, quality and therefore, this virus has a high economic importance. Also, yellowing symptoms on cucurbit plants are similar in their occurrence and aggressiveness either in high temperatures during summer, early autumn and early spring depending on the presence and number of whiteflies population and susceptibility of affected cultivars. Such results confirm those of Abou-Jawdah *et al.* (2000); Rubio *et al.* (2001); Lecoq and Desbiez (2012); Tzanetakis *et al.* (2013); Abrahamian and Abou-Jawdah (2014) and Abrahamian *et al.* (2015) who mentioned that the whitefly-transmitted criniviruses are reported to induce yellowing symptoms and to be the primary cause of significant crop losses in many regions worldwide with tropical, subtropical, arid, and Mediterranean climates.





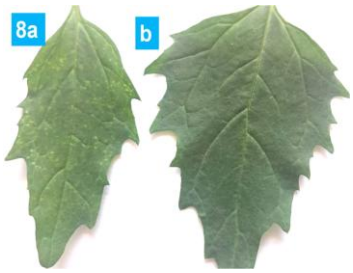


Fig. 1. CYSDV-naturally infected and whiteflies transmission symptoms on cucumber cv. El-Safa plants:(a) Healthy leaf, (b) interveinal yellowing (left) and green veins (right), (c) yellowing and green veins on leaves, 3-weeks post-inoculation by viruliferous whiteflies.

Fig. 2. CYSDV-naturally infected and whiteflies transmission symptoms on squash cv. Escandarani plants:(a) Healthy leaf, (b) interveinal yellowing and green veins, (c) yellowing and green veins on leaves, 3-weeks after whiteflies inoculation.

Fig. 3. CYSDV-naturally infected and whiteflies transmission symptoms on watermelon cv. Giza plants:(a) Healthy leaf, (b) yellowing and green veins, (c) yellowing and green veins on leaves 3-weeks post-inoculation by viruliferous whiteflies.

Fig. 4. CYSDV-naturally infected and whiteflies transmission symptoms on melon cv. Al Shams plants:(a) Healthy leaf, (b) yellowing and green veins.

Fig. 5. Yellowing and green veins symptoms of CYSDV-naturally infected pumpkin plants.

Fig. 6. (a) CYSDV symptoms of yellowing and green veins on naturally infected okra plants (*Abelmoschus esculentus*), (b) Healthy leaf.

Fig. 7. (a) CYSDV-naturally infected *Malva parviflora* exhibited yellowing and green veins, (b) Healthy leaf.

Fig. 8. (a) CYSDV-naturally infected *Chenopodium album* reacted with yellowing and green veins, (b) Healthy leaf.

Virus-vector relationships:-

In a preliminary experiment, the assumed virus could not be transmitted mechanically, but successful whiteflies transmission was obtained onto healthy cucurbit plants. In whiteflies transmission experiments, the maximum transmission efficiency (86%) was recorded, when applying fifteen insects in each treatment to transmit CYSDV after 48h for both AAP and IAP (Table 1 & Figs. 1-5). In order to determine the percentages of natural infection and whiteflies transmission DAS- and indirect ELISA were utilized. Data presented in Table (1) reveal that the highest percentages reached to 93 and 86% respectively, in CYSDV-naturally infected squash cv. Escandarani plants and post-inoculation whiteflies. Whilst, in cucumber varieties the percentages of natural infection and whiteflies transmission ranged between 20-80% and 20-86%, respectively. The lowest percentages were recorded in watermelon and pumpkin being 6% and 13% respectively, in natural infection and whiteflies transmission. The obtained results are consistence with those recorded by whitefly-transmitted criniviruses as have been mentioned on cucurbit crops in Spain (Marco and Aranda, 2005), on cucumber in Lebanon (Abrahamian and Abou-Jawdah, 2014). Moreover, these results confirm those of Abou-Jawdah *et al.* (2000); Aguilar *et al.* (2006); Kuo *et al.* (2007); Wintermantel*et al.* (2009) and Abrahamian *et al.* (2015) who revealed that CYSDV is the major yellowing virus in greenhouses in Lebanon and Spain respectively, and has become the prevalent virus in protected cucurbit crops, where up to 100% of plants are frequently infected. Moreover, several investigators mentioned that the impact of the virus outbreak was immediate and widespread, with nearly 100% infection of cucurbit crops depending on the season of plantation. The epidemics coincided with gradually increasing populations of *B. tabaci*, biotype B, the whitefly vector responsible for transmission of CYSDV. It is transmitted by at least two whitefly species, *B. tabaci* (Genn.) and *B. argentifolii* (Celix *et al.*, 1996; Livieratos *et al.*, 1999; Martelli *et al.*, 2000 and Wintermantel *et al.*, 2009). Further, Wintermantel and Wisler (2006) in their studies on *Tomato chlorosis crinivirus* (ToCV) revealed that the transmission efficiency varies among whitefly species, with *T. abutilonea* and *B. tabaci* biotype B, highly efficient vectors, yielding high rates of transmission, whereas *B. tabaci* biotype A and *T. vaporariorum* transmit (ToCV) with much lower efficiency. *B. tabaci* biotype Q is also an efficient vector, and has emerged as the predominant vector in Southern Europe. Each vector also differs in its ability to retain the virus, with *T. abutilonea*

able to transmit for up to 5 days following virus acquisition, whereas *B. tabaci* biotype B loses its ability to transmit ToCV after 3days. *B. tabaci* biotype A and *T. vaporariorum* lose their transmissibility after only one day.

Concerning experimental of non-cucurbit plants as reservoir hosts, the survey proved that the host range of CYSDV in Egypt included member of non-cucurbits plants within the families *Amaranthaceae, Chenopodiaceae, Leguminosae (Fabaceae), Malvaceae, and Solanaceae*. Symptoms on infected plants ranged from mild to extremely severe. The results were recorded after whitefly inoculation from CYSDV-infected cucumber to healthy okra plants (*Abelmoschus esculent*us L.) and *Malva parviflora* L. and other hosts as mentioned in Table (2). Symptoms of back inoculation were nearly similar to those reported for CYSDV. These results were confirmed by EM to insure the typical CYSDV exhibited symptoms and virus particles either in the test (non-cucurbits) plants or cucurbit one. The host range for CYSDV upon whitefly inoculation and back inoculation was nearly similar to those reported for CYSDV (Wintermantel *et al*., 2009 and Okuda *et al.*, 2010). Obtained results indicate that the most efficient hosts of CYSDV are predominantly members of the *Cucurbitaceae* and non-cucurbit hosts play an important role for survival and maintenance of CYSDV in the environment of the Egypt where these hosts or their relatives grow near cucurbit plantations. As well as, they are considered as potential hosts (reservoirs) for maintenance and transmission of a wide array of criniviruses. Conversely, several investigators revealed that CYSDV has a narrow host range limited to species of the family *Cucurbitaceae* and is confined to phloem-associated cells (Hassan and Duffus, 1990; Célix *et al*., 1996; Marco *et al.*, 2003). Recently, CYSDV has become spread worldwide and many workers registered wide range of hosts belonged to non-cucurbit plants (Marco and Aranda 2005; Wintermantel *et al.*, 2009 and Okuda *et al.*, 2010). Nowadays, Wintermantel *et al.* (2016) recorded that the virus incidence in fall melon fields was 100% in each year. Also, they suggested that the virus had become established in native vegetation, weeds, and other crop species, and represented an increasing threat to melon production in the Southwestern United States.

Table 1. Evaluation of the produced antibody (CYSDV-Pab) in determining percentages of CYSDV-infection in different cucurbit crops through two successive seasons (2011-2012) using indirect- & DAS-ELISA.

Crop	Cultivar	Symptoms	*Average percentages of CYSDVinfection in two successive seasons (2011-2012) for:		Detection methods	
			% Natural infection	% Insect transmission	DAS ELISA	Indirect ELISA
Cucumber	El Safa	SS	10/15 (66%)	12/15 (80%)	+	NT
	El Sahm	SY	7/15 (46%)	13/15 (86%)	+	NT
	Ku-12	MS	3/15 (20%)	3/15 (20%)	+	NT
	Hybrid 173	YS	8/15 (53%)	9/15 (60%)	+	NT
	Ku-10	MS	11/15 (73%)	13/15 (86%)	+	NT
	Al Zaem	YS	12/15 (80%)	11/15 (73%)	+	NT
	Sina-2	MS	9/15 (60%)	10/15 (66%)	+	NT
	Sina-1	SY	13/15 (86%)	12/15 (80%)	+	NT
	Kuck	MS	8/15 (53%)	10/15 (66%)	+	NT
	Monier	SY	9/15 (60%)	10/15 (66%)	+	NT
	Hybrid	MS	10/15 (66%)	11/15 (73%)	+	NT
	Beta-Alpha	YS	12/15 (80%)	13/15 (86%)	+	NT
Cantaloupe	Al Shams	MS	3/15 (20%)	5/15 (33%)	NT	+
Squash	Escandarani	Y&SS	14/15 (93%)	13/15 (86%)	NT	+
	Mabrouka	Y&SS	6/15 (40%)	8/15 (53%)	NT	+
Watermelon	Hybrid	Sls	2/15 (13%)	3/15 (20%)	NT	+
	Giza-1	MS	1/15 (6%)	2/15 (13%)	NT	+
Pumpkin	Pumpkin	Sls	1/15 (6%)	2/15 (13%)	NT	+

*Number infected plants / number total plants
Sls = Slight symptoms
SY = Severe yellowing
NT = Not tested
\+ = Positive results
MS = Mild symptoms
SS = Severe stunting
Y&SS = Yellowing & Severe stunting
YS = Yellowing stunting

Virus examination using electron microscopy:-

Examination of grids floated on infectious sap extracts using dip preparation of CYSDV-infected cucumber leaves observed very few flexuous particles. Also, examination of crude sap extracts of CYSDV-naturally infected and whitefly inoculated non-cucurbit plants as shown in Table (2) which observed few flexuous particles. CYSDV has flexuous filamentous virions approximately 850 nm in length (Fig. 9) that are similar to those examined by Cotillon *et al.* (2005) who observed non-decorated of CYSDV particles in a crude extract of the infected cucumber leaf, as well as decorated particles when using the immuno-electron microscopy with antiserum.

Table 2. Survey and Detection of CYSDV in non-cucurbit plant species grown around cucurbit plants in the greenhouses and open fields.

Tested plant	CYSDV symptoms of:		Detection by electron microscope
	Natural infection	Whitefly inoculation	
Amaranthaceae **:**			
Amaranthus retroflexus (Redroot pigweed)	**Y**	**Y**	**+**
Chenopodiaceae **:**			
Chenopodium murale (Nettle leaf goosefoot)	**CS**	**CS**	**+**
Leguminosae (***Fabaceae***) **:**			
Phaseolus vulgaris L. cv. Top Crop	**IC**	**IC**	**+**
Malvaceae **:**			
Abelmoschus esculentus L.	**Y&SGV**	**Y&SGV**	**+**
Malva parviflora L.	**Y&SGV**	**Y&SGV**	**+**
Solanaceae **:**			
Capsicum annuum L. cv. Califonia Wonder	**0**	**0**	**NT**
Datura stramonium L.	**Y**	**Y**	**+**
Solanum lycopersicum L. cv. Super Marmand	**0**	**0**	**NT**

CS = Chlorotic spots, IC= Interveinal clorosis GV = Green Veins
\+ = Positive reaction - =Negative reaction Y = Yellowing
NT = Not tested

Purification of CYSDV and antiserum production (Polyclonal antibody):-

The objective of antiserum production (CYSDV-Pab) was to detect the dissemination of CYSDV-infections and its relationship with yellowing phenomenon in cucurbit cultivations (either in fields and greenhouses) by DAS-, indirect ELISA, TBIA and DBIA. Antiserum was collected at weekly intervals start from the 4th week after the first injection. The maximum virus yield of purified γ-globulin 20mg/200g fresh weight of cucumber leaves was obtained. The UV spectrum of the major fraction is characteristic for nucleoproteins and possesses a ratio A_{260} / A_{240} was 1.3 (Fig. 10). Result indicates that only one peak appeared when purify ed virus was separated after 2h. Four dilutions of both γ-globulin (IgG) and γ-globulin conjugate (IgG-Ap) were used in each of citrate or general buffer. In DAS ELISA, the dilutions of 1/250, 1/500 in both of γ-globulin (IgG) and γ-globulin conjugate (IgG-Ap), respectively gave the best results of optical density values (O.D.) when citrate buffer was used (Fig. 11). Whilst in case of using general buffer, the dilution of 1/250 in both of γ-globulin and γ-globulin conjugate was the best than the other three dilutions to obtain similar O.D values for antigen detection. DAS-ELISA tests were carried out with leaves of infected cucumbers taken at three different levels, from the bottom to the apex. High absorbance values at 405 nm (A_{405nm}) were obtained, especially with young leaves showing fewer symptoms than older leaves. No cross reaction was observed with extracts from uninfected cucumber. In this respect, the obtained results confirm those reported by Monis and Bestwik (1996); Hull (2000) and Kreuze *et al.* (2002) who mentioned in their works on the progression of CYSDV infections differed from other viruses in which younger leaves had the higher virus titers early after inoculation, and was similar to other closteroviruses in which older leaves had higher virus titers.

The result reveals the affinity (strength) of the reaction between antigen, γ-globulin and γ-globulin conjugate. In the standard DAS–ELISA, even though the CYSDV-Pab allowed differentiation between infected and healthy samples, the ratio of readings at A_{405nm} was only 5-7x (infected/healthy) and required a minimum 2h in substrate incubation at 37°C. In indirect plate-trapped ELISA, 2h incubation with the substrate was enough to produce a strong reaction with extracts of CYSDV-infected plants when using an antibody dilution of 1:1000, and antigen incubation at overnight at 4°C. Indirect ELISA gave high absorbance values within 2h in substrate incubation. As mentioned by Hourani and Abou-Jawdah (2003) who explained that the virus is restricted to the phloem of infected plants and generally found at very low concentrations, therefore it was very difficult to purify for antiserum production. Whilst

in the present investigation, the induced antiserum was a high titer. The aforementioned results of the induced antiserum showed that CYSDV-Pab can be successfully used in serological tests such DAS- and indirect ELISA, as well as proved to be the most appropriate for performing routine tests in detecting high numbers of CYSDV-naturally infected cucurbits or non-cucurbits plant. Similar results had been observed by several investigators working on CYSDV (Livieratos *et al.*, 1999; Hourani and Abou-Jawdah, 2003) or other related viruses classified under family *Closteroviridae* (Nikolaeva *et al.*, 1995; Ling *et al.*, 2000 and Sequeira & Nolasco 2002). Further, Hourani & Abou-Jawdah (2003) and Cotillon *et al.* (2005) found the importance of polyvinylpyrrolidone (PVP) and alkaline pH of the extraction buffers, as well as Tween 20 in obtaining good results during ELISA performance. Relevant to the present work result obtained by Cotillon *et al.* (2005) who mentioned that A_{405nm} values obtained in DAS-ELISA tests with the purified polyclonal antibodies confirmed that these antibodies detect the virus as effectively in symptomless young leaves as in old leaves showing severe yellowing. In contrast, Marco *et al.* (2003) found that in susceptible accessions of melon, cucumber, marrow, and squash, CYSDV RNA accumulation peaked during the first to second weeks after inoculation in the first to third leaves above the inoculated one; younger leaves showed very low or undetectable levels of CYSDV. This could reflect a defect of phloem cells of young leaves for the support of the replication or the movement of closteroviruses, as has been hypothesized at least in one other occasion.

Evaluation of cucumber varietal reaction using the induced antibody:-

Twelve cucumber cultivars were evaluated for their susceptibility by DAS-ELISA using the induced antibody at four different incubation periods *i.e.*, 10, 30 min, 1h and overnight with paranitrophenyl phosphate (PNPP) substrate. Data presented in Fig. (12) reveal the absorbance readings (optical density "O.D.") values after incubation periods of 1h and overnight were nearly consistence. After incubation periods of 1h and overnight, the highest value readings were recorded for the cultivars Sina 2, Ku-10, Monier and Hybrid-173 compared with the positive sample. While, the lowest O.D. values were recorded Sina 1, El Sham and Ku-12. ELISA readings (A_{405}) at Sina 1, El Sham and Ku-12 confirmed that virus titers in the tolerant were lower than in the susceptible controls. On the other hand, the results showed that although Sina 2, Ku-10 gave high reading values but they reacted with mild symptoms (interveinal chlorotic spots), compared with Monier and Hybrid-173 which gave similar high readings and reacted with severe symptoms (severe yellowing). Regarding results in Table (1) revealed that the lowest percent (20%) was recorded for cucumber plants cv. Ku-12 in both of natural infection or whiteflies transmission. These results indicate that Ku-12, El-Safa, Kuck, Sin-1 El Sahm and Hybrid plants might be carried a resistance to CYSDV multiplication or spread in the plant. Also, the differences between the reaction of the various cultivars could be due to genetically or physiological differences. So, we recommend for plantation the tolerant cultivars to prevent virus dissemination and its changing to epidemic disease. This result resembles those obtained by Eid *et al.* (2006) who mentioned in their studies on tolerance in cucumber to CYSDV that the virus resistance can be defined as a reduction in symptoms associated with reduced virus concentrations, whereas tolerance often is defined as a reduction of symptoms with slight or no reduction in virus concentrations. None of the cucumber accessions showed immunity unlike that reported for melon. Further, they found that both DBIA and ELISA readings at 18th weeks post-inoculation revealed that accessions PI-605923, Ames 3950, Ames 13334, PI-177364, and PI-211589 had much lower virus concentrations than susceptible accessions. Also, cucumber accessions PI-211589, PI-605923, and Ames 13334 showed tolerance to CYSDV because they developed less severe symptoms in three different tests, and accumulated lower virus titers compared with susceptible accessions. Whilst, accessions PIs-177364, 279807, and 293432 and NSL 5746 also may be promising sources of tolerance under moderate disease pressure.

Similar trend was also observed by Marco *et al.* (2003) who mentioned that the melon accession namely C-105 remained CYSDV-free for up to 6 weeks after whitefly inoculation. Virus titer was very low level when detected by RT-PCR in whitefly-inoculated leaves, and therefore, multiplication or spread of CYSDV appeared to remain restricted to the inoculated leaves. Also, López-Sesé and Gómez-Guillamón (2000); Marco *et al.* (2003) and Eid *et al.* (2006) in their investigation on serological assays showed that CYSDV is unevenly distributed within the cucumber plant and that middle leaves are better than the top and bottom leaves to compare relative virus concentrations in the plant. Similar results were reported for CYSDV in melon. Resistance to CYSDV in the melon 'TGR-1551' was controlled by a single dominant gene which may restrict virus movement in the vascular system of the plants or prevent high levels of virus accumulation. Therefore, the resistance mechanism may involve a restriction of the virus movement in the vascular system of the plants and/or prevention of high levels of virus accumulation. Furthermore, López-Sesé and Gómez-Guillamón (2000) and McCreight and Wintermantel (2011) found that the use of genetically resistant cultivars was a good option for CYSDV control, but no resistant cultivar was commercially available. They also reported that the existence of possible sources for natural resistance or

tolerance to CYSDV. Moreover, Fraser (1990) and Maule *et al.* (2000) reported that the possibility of low level of CYSDV multiplication in C-105 is not enough to trigger the cascade of events associated with symptom induction and lack of symptom expression has been shown to be accompanied by reduced levels of virus multiplication in other cases.

CYSDV-detection by TBIA and DBIA techniques:-

Concerning TBIA approach, the best dilution of induced rabbit poylclonal antiserum (primary antibody) cross absorbed and AP-labeled secondary antibody (goatanti-rabbit) was about 1:1000. TBIA detected CYSDV antigen in infected cucumber cv. El-Safa plants in both leaves and petioles (Fig 12A). Data indicated that TBIA was able to detect virus in both naturally infected plants. The result revealed that although DAS-ELISA is the most common serologic technique used in virus detection, but TBIA is highly recommended for large scales surveys of CYSDV and others viruses worldwide and specially in the developing countries. The obtained results are in agreement with those obtained by Hourani and Abou-Jawdah (2003) and Abou-Jawdah *et al.* (2008) who mentioned that TBIA was the most sensitive serological method used because it allowed CYSDV detection within 5-6 days post-inoculation. Also, they found that TBIA is recommended for rapid detection and comparison of CYSDV movement between resistant and susceptible cucumber accessions.

In addition to TBIA, applying DBIA at dilution of 1:1000 for induced rabbit poylclonal antiserum cross absorbed, whilst the dilution of anti-rabbit alkaline phosphatase conjugate was about 1:2000. It is worth mentioning that the induced antiserum (CYSDV-Pab) successfully detected CYSDV antigen in naturally infected plants (Fig 12B). DBIA save time and antibodies, since the antibody solution can be reused several times. These results confirm the efficiency of DBIA for detection criniviruses as discussed by Eid *et al.* (2006) and Abou-Jawdah *et al.* (2008), as well as other viruses (Makkouk *et al.*, 1993; James and Mukerje, 1996; Ghanem, 2003 and Ajlan *et al.* 2007). In conclusion, DBIA and TBIA proved to be effective in differentiating between healthy and infected samples, faster, sensitive and reliable techniques for the detection CYSDV in cucumber leaf petioles.

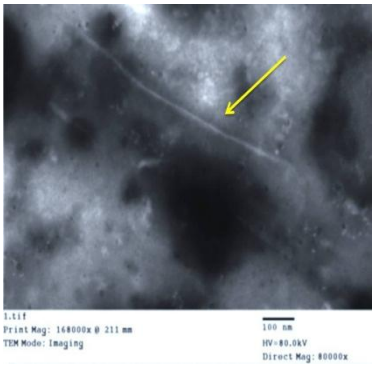


Fig.9 An electron micrograph of purified virus preparation showing CYSDV particles 850nm in length.

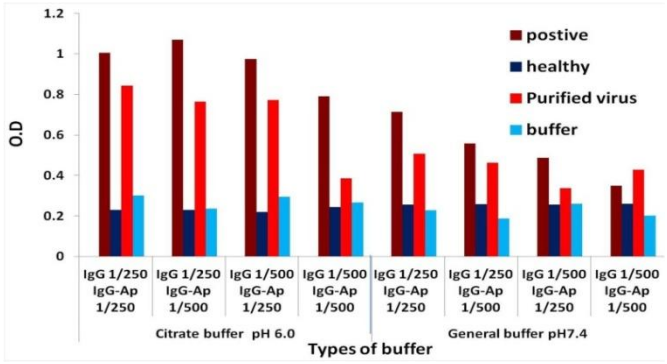


Fig.11 Determination of the optimal concentration of produced antiserum (IgG & IgG-AP) for detecting CYSDV-infected tissues using two different buffers.

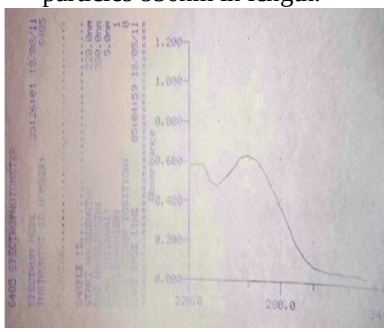


Fig.10 Ultra violet absorption spectrum of the supernatant containing CYSDV-isolate.

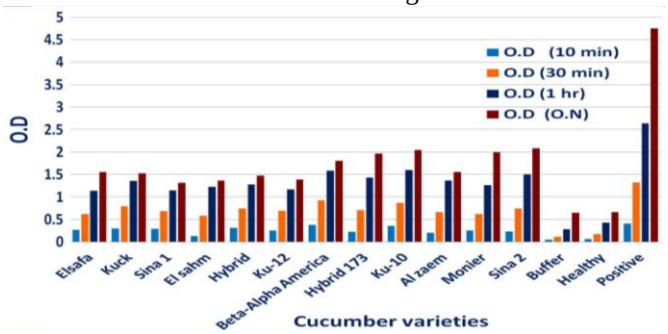


Fig.12 Evaluation of cucumber varietal reaction against CYSDV-infection using the produced antibody (CYSDV-Pab) in DAS-ELISA at different incubation periods with paranitrophenyl phosphate (PNPP) substrate.

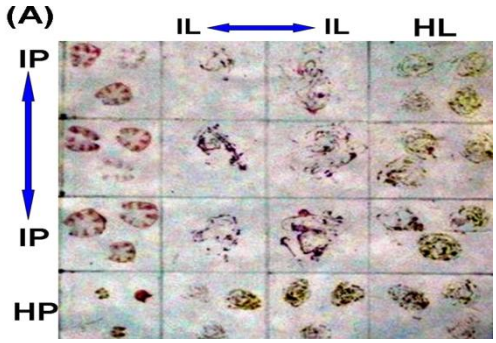


Fig.12 A TBIA detection of CYSDV in petioles and midrib of cucumber cv. El Safa plants HL = Healthy leaf, IL = Infected leaf, HP = Healthy petioles andIP= Infected petioles.

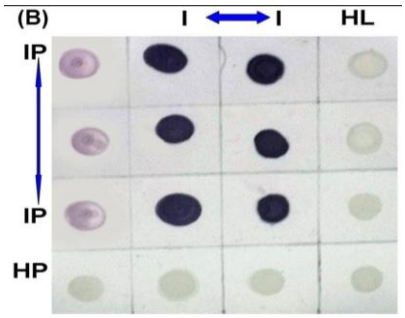


Fig.12B DBIA detection of CYSDV in cucumber cv. El Safa plants, HL = Extracted sap from healthy midrib, I = infected midrib, HP = Extracted sap from healthy petioles. and IP = infected petioles.

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