Fidan, H. Unlu, M. Unlu A.and Yilmaz M.A. 2012 Cucurbit Yellow Stunting Disorders (CYSDV) and Cucumber Vein Yellowing Virus (CVYV) diseases on melon and cucumber in Turkey Xth EUCARPIA International Meeting on Genetics and Breeding of Cucurbitaceae 2012 p-755-762

Cucurbit Yellow Stunting Disorders (CYSDV) and *Cucumber Vein Yellowing Virus* (CVYV) Diseases on Melon and Cucumber in Turkey

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Key words; CYSDV, CVYV, *Bemisia tabaci*, Biotype, Cucumber, Melon.

Abstract

The aim of this research was to determine the causal organisms of yellowing and vein clearing type of symptoms of melon and cucumber plants growing under greenhouse conditions, and which *Bemisia tabaci* biotypes are involved in transmission of viruses in Adana, Mersin and Antalya provinces. For this purposes, the diseases surveys were periodically made in 2010 and 2011, and whitefly vectors collected on the young leaves of the hosts from different district of cucurbits growing area. The virus infected plants were tested against to cucurbit viruses by PCR. The result of work suggested that CVYV and CYSDV are responsible for the disorders of the yellowing type of symptoms. The infected plants showed mainly interveinal chlorosis and yellowing type of nutrition deficiency symptoms on the leaves. Among the collected 134 melon samples, 63 and 24 plants were found to be infected by CYSDV and CVYV. In the case of cucumber samples, CYSDV and CYVV were detected on the 82 and 12 plants out of 110 cucumber plants. The collected whitefly-vector from the infected cucumber and melon plants were tested by PCR. The assay showed that *B. tabaci* Biotype B (92%) efficiently transmits the viruses but Q biotype (8%) poorly transmitted the viruses. The study of PCR clearly pointed out that individual whitefly is a harbour of viruses and transmits the viruses. DNA fragments were directly sequenced and compared with CYSDV and CVYV isolates with that of Israel, USA and Spain isolates. It was concluded that 96% of CYSDV (AY580985, FJ492808) and 95% of CYVV isolates nucleotide identity with the coat protein gene with Israel, USA and Spanish isolates.

Introduction

Virus diseases are a worldwide problem of (*Cucurbitaceae*) and a major limiting factor for cucurbit production. Cucurbit viruses can infect many plants belonging to different genera and families, and only a few viruses are restricted to cucurbits species. Cucurbit viruses reduce the plant vigour, number of flowers, and fruits (Luis-Arteaga *et al.* 1998). Among approximately 35 viruses infecting cucurbits worldwide, more than ten viruses have been reported in Turkey (Provvidenti, 1996) (Yilmaz *et al*., 1989). The important cucurbit viruses are the aphid-borne *Cucumber mosaic virus* (CMV), *Papaya ringspot virus* (PRSV-W), *Watermelon mosaic virus*

(WMV), *Zucchini yellow mosaic virus* (ZYMV), *Cucurbit aphid-borne yellows virus* (CABYV), the whitefly-borne *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Beet pseudo-yellows virus* (BPYV), the beetle-transmitted *Squash mosaic virus* (SqMV), and *Melon necrotic spot virus* (MNSV, genus Carmovirus, family Tombusviridae) (Zitter et al. (1996), Fidan et al. (2010)) During earlier studies whitefly-borne cucumber viruses were biologically detected but the distribution and incidence of the viruses were not studied.

During survey, leaf mottling, mosaic, vein banding, vein clearing; leaf and fruit malformation symptoms are most frequently noticed in field-grown cucumber and melon. In greenhouse conditions however yellowing type of symptoms were observed on cucumber which often attributed to Mg++, Fe and Mn++ deficiencies (Papyiannis et al. 2005).

Cucumber vein yellowing virus (CVYV) being responsible for important economical losses in several crops, causes a whitefly-transmitted disease in cucurbits. The virus has been taxonomically included in the genus Ipomovirus, family of Potyviridae (Lecoq et al., 2000). First, CVYV is transmitted by *Bemisia tabaci* in a semi-persistent manner (Mansour and Musa, 1993), a feature shared with other members of the genus (Jones, 2003). Finally, molecular data derived from comparison of a partial sequence of the 3' region of the viral genome of the Israelian isolate CVYV-Isr have confirmed the closest relationship of CVYV with Ipomoviruses (Lecoq et al., 2000). CVYV naturally infects cucumber (Cohen & Nitzany, 1960), melon (Yilmaz *et al*., 1989), watermelon (Janssen & Cuadrado, 2002) and courgette. Wild cucurbits are also reported as hosts in Jordan (Mansour & Al-Musa, 1993) CVYV is a positive-sense single-stranded RNA virus member of the family Potyviridae. The genomic RNA of potyviruses is translated into a single polyprotein that is proteolytically processed by three virus encoded proteases (Sweiss et al 2007).

B. tabaci became viruliferous after an acquisition period of 30 min and transmission reached 51% after a 4-h feeding period. Extending the acquisition time had little effect on the transmission rate. CVYV was retained by its vector for no longer than 4–6 h (Harpaz & Cohen, 1965) and the latent period was at least 75 min. Transmission by viruliferous whiteflies occurred after a 15-min inoculation feeding period. The highest level of transmission (57.5%) was reached after a 4-h inoculation period. The aphids *Aphis gossypii* and *Myzus persicae* are not vectors of CYSDV and CVYV (Cohen & Nitzany, 1960).

CYSDV is a member of the genus *Crinivirus* (family Closteroviridae) efficiently transmitted by *B. tabaci*, biotypes-B (*B. argentifolii*) and Q (Berdiales et al., 1999). The natural host range of CYSDV is limited to the family of Cucurbitiacea mainly on cucumber, melon, watermelon and squash. The severe yellowing induced by CYSDV drastically affects fruit number and weight leading to a 30–50% yield reduction (Abou-Jawdah et al., 2000). First detection of CYSDV was in the United Arab Emirates in 1982 (Hassan and Duffus, 1991) where it remains in epidemic proportions (Duffus, 1995). CYSDV has since spread throughout the Mediterranean region (Celix et al., 1996) including Egypt, Israel, Jordan, Spain, Turkey, Lebanon, Portugal, and Morroco where it has caused major economic damage to cucurbit crops (Papyiannis et al. 2005). CYSDV has also been introduced to North America, specifically the Rio Grande Valley of southern Texas and northern Mexico (Kao et al., 2000).

Materials and Methods

The survey and sample collection were carried out Mediterranean region during 2010–2012 in the major cucumber and melon growing area of Adana, Mersin and Antalya provinces (Table 1).

The survey and sample collection were carried out in Mediterranean region of Turkey during 2010–2012 in the major cucumber and melon growing area of Adana, Mersin and Antalya provinces (Table 1). A total of 244 plant samples showing virus-like symptoms (cucumber from 66 greenhouses and melon from 55 greenhouses) were collected from 34 different locations. At the same time a total of 340 adult whitefly individuals were collected on infected cucumber and melon plants inwhich same greenhouses.

Each sample consisted of the youngest fully developed leaf from plants exhibiting chlorosis, vein clearing or yellowing, and fruit discolorations and deformation (Figure 2A, B). All samples were placed in plastic bags, stored at 4ºC, and tested within 1–3 days for virus infection either serologically [double-antibody sandwich enzyme-linked immunosorbent assay (DASELISA)] or by reverse transcription polymerase chain reaction (RT–PCR).

All plant samples (244) were tested by DAS-ELISA for the presence of CYSDV. The 244 samples that exhibited yellowing or vein clearing symptoms and originating from cucumber crops were also tested by RT–PCR for the presence of CYSDV, BPYV, CABYV and CVYV. A sample was considered virus-positive if its A_{405} nm value was greater than three times that of healthy plant extract.

Plant genomic RNA was isolated by freezing leaf in liquid nitrogen and grinding the tissue to a fine powder using a minipestle in an Eppendorf tube. After addition od 566 µl of extraction buffer (100mM Tris8.0, 50 mM EDTA, 500mM NaCl, 10 mM mercapto-ethanol and 1% sodium dodecyl sulfate (SDS)) the tubes were vortexed and incubated at 65 ºC for 10 min; then 160 µl of 5 M M potassium acetate was added to each tube, followed by vortexing and centrifugation for 15 min. The supernatant was removed and RNA precipitated with isoprooanol. The pellet was washed twice in 70% EtOH and resuspended in 400 µl of sterile water. One hundred nanograms of RNA was used for the each polymerase chain reaction (PCR)

Whitefly DNA isolation: Kill insects by freezing in microfuge tube. Add 10uL 0.5M NaOH to each tube. Crush insect with pipette or Kontes pestle for 30 seconds. Add 30uL 1M Tris-HCl buffer (containing 1% SDS and 20mM EDTA, pH8.0), mix well. Incubate solution at 65 C for 15 minutes, centrifuge at 12,000 rpm for 10 minutes. Transfer 30uL of supernatant to new tube. Add 30uL 95% ethanol, mix, and freeze for 30 minutes at -20ºC. Precipitate DNA by centrifugation at 12,000 rpm for 10 minutes. Dry pellet at 72 C and resuspend in 30uL 1X TE((Potter et al. 2003) (Kadhi et al.2008)).

Reverse transcription polymerase chain reaction (RT-PCR) Primers used in this study and the expected length of each amplicon are presented in Table 2.

First-strand cDNA synthesis was carried out Maloney Murine Leukemia Virus (M-MLV) reverse transcriptase (FERMANTAS,) using the antisense primers (Table 2). Two-step RT-PCR was performed according to the procedures described previously (Papyiannis et al. 2005). PCRs were performed using Taq Polymerase (Fermantas DreamTaq™ Green DNA Polymerase), according to manufacturer's recommendations and 0.2 µM of each forwards primers and Reverse specific primers respectively. The temperature regime for amplification reactions was as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles of 95°C for 30 s, 54°C (CYSDV and BPYV), 55°C for CVYV, 62°C CABYV for 30 s and 72°C for 30 seconds. The final extension was at 72°C for 7 min. A GeneAmp 9700 thermocycler (Eppendorf Mastercycler Gradient PCR Thermocyler) was used for RT-PCR amplifications.

PCR reaction for whitefly. The basic mixture consists of 10.4 µl sterile distilled water (milli Q), 2 µl of 10Xpcr buffer, 1.4 µl of 50x MgCl2 d), 1 µl of 2.5 µl mM dNTPs, 1 µl of 10 µM Bem 23F, 1 µl of 10 µM Bem 23R, and 0.25 µl dreamTaq (5 uds/µl) in a final volume of 17 µl. The parameters of the thermal cycle are as follows: firstly at 94°C for 5 min, secondly at 94°C for 30

sec, thirdly at 55°C for 1 min, fourthly at 72°C for 1 min, fifthly repeating the cycle steps second through fourth, sixthly at 72°C 40 times for 10 min and lastly storing at 4°C until electrophoresis.

PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The amplicons were then directed sequenced by SENTROMER Company. Sequence analysis was compared by using BioEdit v 7.0.5 programs.

Result and Discussion

Plant samples were tested by ELISA and RT-PCR in order to determine which of the viruses (CVYV, CYSDV, CABYV and BPYV) showing similar symptoms infected the plants.

A total of 244 melon and cucumber were tested by ELISA for CYSDV. Among the tested samples 24 cucumber (22%) and 63 melon (47%) were found to be infected by CYSDV. On the other hand, CABYV and BPYV were not detected on the samples.

Cause of not detecting CABYV could be possible that absence of the vectors (aphids). Difficulties of entering into greenhouses and good pest management against to aphids cause this result. BPYV which is transmitted by *Trialeurodes vaporarium* have not been reported in Mediterranean region.

The coat protein specific for CYSDV cDNA band at 364 bp were obtained from 63 out of 143 melon samples and 12 out of 134 cucumber samples.

By using of ELISA and RT-PCR methods, CYSDV were quite often detected on melon growing in greenhouse. In the RT-PCR tests with primers specific to CVYV, 450 bp cDNA bands were successfully obtained from cucumber samples (82 out of 110) showing CVYV symptoms (Figure1B). The virus was detected among 74% of collected samples. On the other hand, infection rate in the melons was found to be 9 %. Among the collected 340 whitefly from the samples, *B tabaci* Biotype B (92%) were found in the using of PCR tests with co dominant primer Bem23. However, *B tabaci* Biotype Q were found 8 % at the same hosts by using of the Bem23 primer (Figure1C). In order to investigate the presence of virus in the collected whiteflies samples, DNA was isolated from 1, 5 and 10 individuals. CYVY and CYSDV were detected even in a single white fly by using of RT-PCR however if the number of whitefly increased to the 10 individuals, the success of isolation of DNA also increased.

PCR the assay showed that *B.tabaci* Biotype B (92 %) efficiently transmits the viruses but Q biotype (8%) poorly transmitted the viruses. The study of PCR clearly pointed out that individual whitefly is harbour of viruses and transmits the viruses. DNA fragments were directly sequenced and compared with CYSDV and CVYV isolates with that of Israel, USA and Spain isolates. It was concluded that 96 % of CYSDV (AY580985, FJ492808) and 95 % of CYVV isolates nucleotide identity with the coat protein gene from Israel, USA and Spanish isolates.

These results are important for the management of the yellowing diseases (CYSDV and CYVV) on cucurbits in Turkey. Chemical control of vectors (whiteflies) has not been effective to prevent to spread of these transmitted viruses by whiteflies therefore genetic resistance varieties has to develop in melon and cucumber germplasm as a means of controlling these diseases.

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Table 1: Survey and collection of samples at major cucurbits growing areas in Mediterranean region.

	CYSDV		CVYV	

	Cucumber	Melon	Cucumber	Melon
Adana	6[a]/27[b]	12[a] /41[b]	22[a] /27[b]	2[a] /41[b]
Mersin	7/39	22/48	29/39	7/48
Antalya	11/44	29/45	31/44	3/45

[a] Number of infected plants.

[b] Number of collected samples

Table 2: Primers used in RT-PCR, and predicted amplification size for detection of CVYV, CYSDV, BPYV and CABYV

Primer	Sequences	Amplicon size bp
CYSDVF	AGTGACATGCCTAACTGTTACTT	364bp
CYSDVR	ATAGCTGCTGCAGATGGTTC	
CVYVF	AGCTAGCGCGTATGGGGTGAC	450 bp
CVYVR	GCGCCGCAAGTGCAAATAAAT	
CABYVF	GAATACGGTCGCGGCTAGAAATC	600bp
CABYVR	CTATTTCGGGTTCTGGACCTGGC	
BPYVF	TCGAAAGTCCAACAAGACGT	251bp
BPYVR	CTGATGGTGCGCGAGTG	
Bem 23F	CGGAGCTTGCGCCTTAGTC	biotype"B" 220bp
Bem 23R	CGGCTTTATCATAGCTCTCGT	biotype"Q"410bp

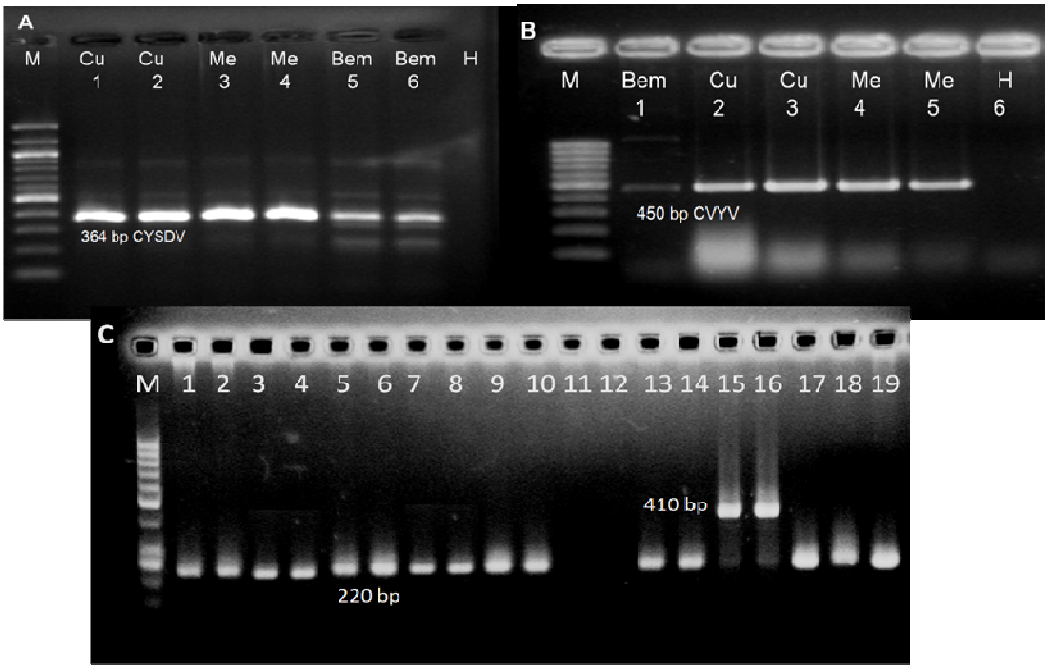


Figure 1. A: Reverse-transcription polymerase chain reaction (RT-PCR) using primer set CYSDVF-R for detecting CYSDV in composite Line 1-2; Cucumber plants Line 3-4; Melon plants Line 5-6; Individual B tabaci PCR amplicons of 364 bp are indicated H: Negative Control.

B: RT-PCR using primer set CVYVF-R for detecting CYSDV in composite. Line1**;** Individual B tabaci PCR amplicons of 364 bp are indicated Line 2-3; Cucumber plants Line 4- 5; Melon plants Line 6 H; Negative Control.

C Line M: molecular weight marker (50 bp DNA ladder). Lines 1-10; Adult of biotype B of *Bemisia tabaci*, showing a band of 220 bp. Lines11–12; DNA free control Lines15-16: Adult of biotype Q of *Bemisia tabaci*, showing a band of 410 bp.



Figure 2: A; Cucumber plant infected with CVYV showing vein clearing and yellowing symptoms. B: yellowing symptoms on Melon C; cucumber plant infected with CYSDV showing typical symptoms on the older leaves.