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RESEARCH PAPERS

The status of *Cucumber vein yellowing virus* in Iran

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Summary. Yellowing symptoms of greenhouse- and open field-grown cucurbit crops are becoming increasingly important in many cucurbit growing regions of the world, and particularly in Iran. A survey was conducted from 2011 to 2012 in eight major cucurbit growing regions in Iran. Yellowing and specifically vein clearing symptoms were observed in many cucumber plants grown in greenhouses and open fields, suggesting the presence of *Cucumber vein yellowing virus* (CVYV, genus *Ipomovirus*, family *Potyviridae*). The identification of CVYV was carried out with a specific triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and reverse transcription (RT)-PCR. CVYV was detected in 42% of the collected samples, in all surveyed provinces, except Bushehr. CVYV was also detected in melon and cucumber crops grown in open fields. These results indicate that CVYV is widely distributed on these two cucurbit species in the major cucumber growing areas of Iran. CVYV positive samples were also tested, using DAS-ELISA, for the presence of *Cucurbit chlorotic yellows virus* (CCYV) and *Cucurbit yellow stunting disorder virus* (CYSDV), two criniviruses reported previously to occur in Iran. Double or triple infections of CCYV and CYSDV occurred in 49 of 166 of the CVYV-infected plants. The CVYV and CCYV combined infections were more prevalent than CVYV and CYSDV combined infections. TAS-ELISA positive samples were used to mechanically inoculate healthy cucumber plants, and mild vein yellowing was observed on the inoculated leaves. Identical symptoms were also observed on whitefly inoculated healthy cucumber plants. The presence of CVYV in mechanically and whitefly inoculated plants was confirmed by TAS-ELISA and RT-PCR. Sequence analysis revealed that the Iranian isolate of CVYV was more closely related to Spanish isolates than to isolates from Jordan. Phylogenetic analysis showed that CVYV isolates can be divided into two phylogenetic groups (I and II). Despite the close vicinity of Jordan to Iran, the Iranian CVYV isolate clustered with Spanish isolates in group I and not with the Jordanian isolates of group II.

Key words: CVYV, TAS-ELISA, RT-PCR.

Introduction

Cucurbits are affected by at least 59 well-characterized viruses belonging to the major plant virus groups (Lecoq and Desbiez, 2012). Different viruses that may cause yellowing symptoms on cucurbits have been reported in several countries, and they may be transmitted either by aphids (Lecoq *et al*., 1992) or by whiteflies (Wisler *et al*., 1998; Jones, 2003). Yellowing symptoms on leaves of melon (*Cucumis melo*), cucumber (*C. sativus*), and squash (*Cucurbita pepo*, *C. moschata*, and *C. maxima*) are the most prevalent symptoms in greenhouse- and open field-grown cucurbits worldwide (Abou-Jawdah *et al*., 2000). Whitefly-transmitted viruses are reported to be the primary cause of significant crop losses in many regions with tropical, subtropical, arid, and Mediterranean climates (Al-Musa *et al*., 2008). The yellowing symptoms on cucurbits can be mistaken with those caused by high temperatures during summer or early autumn, nutritional disorders or pesticide phytotoxicity (Boubourakas *et al*., 2006). However, the presence of initial foci of plants with symptoms near glasshouse openings (doors and windows), random

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distribution, their rapid spread, the ineffective addition of nutrients and the presence of high whitefly population densities can indicate viral causes of cucurbit yellows diseases (Wisler *et al*., 1998).

Criniviruses are an emerging and expanding genus (family *Closteroviridae*) of semi-persistently (non-circulative) whitefly-vectored plant viruses. Several new species have been identified and characterized within the past years (Wintermantel and Hladky, 2010). *Cucurbit yellow stunting disorder virus* (CYSDV) is a member of this genus and is transmitted by at least two whitefly species, *Bemisia tabaci* (Gennadius) and *B. argentifolii* (Bellows & Perring) (Martelli *et al*., 2000). CYSDV was first identified in the United Arab Emirates in 1982 (Hassan and Duffus, 1991). Since then, it has been reported in other countries, including: Spain (Celix *et al*., 1996), Portugal (Louro *et al*., 2000), Morocco (Dsebiez *et al*., 2000), Lebanon (Abou-Jawdah *et al*., 2000), North America (Kao *et al*., 2000), and Iran (Keshavarz and Izadpanah, 2004), where it is recognized as a rapidly emerging and economically important plant virus (Wisler *et al*., 1998). CYSDV is the major yellowing virus in greenhouses in Lebanon (Abou-Jawdah *et al*., 2000) and it has become the prevalent virus in protected cucurbit crops of south-eastern regions of Spain, where up to 100% of plants are frequently infected (Marco and Aranda, 2005). *Cucurbit chlorotic yellows virus* (CCYV), another cucurbit infecting crinivirus, first emerged in Kumamoto Prefecture in the southwest of Japan (Okuda *et al*., 2010). Furthermore, occurrence of, and yield losses caused by, CCYV have been reported in cucurbits in Taiwan, China, Sudan, Lebanon and Iran (Huang *et al*., 2010; Gu *et al*., 2011; Hamed *et al*., 2011; Abrahamian *et al*., 2012; Bananej *et al*., 2013).

Cucumber vein yellowing virus (CVYV, genus *Ipomovirus*, family *Potyviridae*) (Lecoq *et al*., 2000), causes severe yellowing disease and loss of production in cucurbit crops (Mansour and Al-Musa, 1993). This virus is readily transmitted mechanically and by whiteflies (*B. tabaci*) in a semi-persistent manner (Harpaz and Cohen, 1965). Symptoms induced by most CVYV isolates are vein yellowing of the youngest leaves and stunted growth of plants together with reduced fruit production (Cohen and Nitzany, 1960). CVYV naturally infects melon (*Cucumis melo* L.) (Yilmaz *et al*., 1989), cucumber (*C. sativus* L.) (Cohen and Nitzany, 1960), watermelon (*Citrullus lanatus* L.) (Janssen and Cuadrado, 2001), and squash (*Cucurbita* sp.) (Anonymous, 2001). Wild cucurbits are also reported as hosts in Jordan (Mansour and Al-Musa, 1993). CVYV was originally identified in Israel (Cohen and Nitzany, 1960) and it has subsequently been reported from Jordan (Al-Musa *et al*., 1985), Turkey (Yilmaz *et al*., 1989), Spain (Cuadrado *et al*., 2001), Sudan (Desbiez *et al*., 2001), Portugal (Louro *et al*., 2004), Cyprus (Papayiannis *et al*., 2005), Iran (Bananej *et al*., 2006), France (Lecoq *et al*., 2007), Tunisia (Yakoubi *et al*., 2007) and Lebanon (Abrahamian *et al*., 2013). CVYV was recognized as the most prevalent pathogen affecting cucumber in plastic-covered greenhouses in Jordan (Al Musa *et al*., 1985).

In Iran, cucurbits are the major vegetable crops, ranking first in economic value, second in yield, and third in area. Watermelon, melon, and cucumber are cultivated in ~300,000 ha in different provinces of Iran (http://www.maj.ir, http://faostat3.fao.org/home/index.html). In recent years, cucumber cultivation in greenhouses has been developed in many regions of Iran, and yellowing symptoms are frequently found in many greenhouse-grown cucumber crops. Among the cucurbit viruses in Iran, *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Tomato spotted wilt virus* (TSWV) (Samei *et al*., 2004; Shaabanian *et al*. 2004; Massumi *et al*., 2007), *Tomato leaf curl Palampur virus* (ToLCPMV) (Hessari *et al*. 2010), and *Cucurbit aphid-borne yellows virus* (CABYV) (Salehi *et al*., 2012), have been reported from greenhouse-grown cucumbers. During a survey conducted from 2011–12, CCYV and CYSDV were identified in greenhouse-grown cucumber (*C. sativus* L.) and field-cultivated cucumber, squash (*Cucurbita* sp.) and melon (*C. melo* L.) in Iran (Bananej *et al*., 2013). CVYV was only detected in open field grown cucumbers in one region near Jiroft (Kerman Province) in the south of Iran in July 2002 (Bananej *et al*., 2006).

Given the wide endemic population of *B. tabaci* in different regions of Iran (Javan Moghadam, 1993), the prevalent yellowing symptoms in several cucurbit growing regions in open fields and greenhouses (Bananej, unpublished results), and poor knowledge on the status of whitefly transmitted viruses in cucurbits, particular attention should be paid to these whitefly transmitted viruses in Iran.

In this study, the etiology of vein yellowing disease and geographical distribution of CVYV in cucurbits in different provinces in Iran has been investigated, using serological and molecular methods.

Materials and methods

Surveys, sample collection, and TAS-ELISA

During a one-year survey (2011–2012), cucumber, squash, and melon leaf samples with various symptoms such as leaf yellowing, severe yellowing of older leaves and specifically vein yellowing (Figure 1), typical of a CVYV infection, were collected from 394 cucumber plants grown in greenhouses and 107 open field cucumber, squash, and melon crops, from eight different provinces of Iran: Semnan, Tehran, Isfahan, Yazd, Kerman, Fars, Hormozgan, and Bushehr (Figure 2, Table 1). All samples were tested for the presence of CVYV by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA), using a commercially available test kit (AS-0997; DSMZ) following the manufacturer's instructions.

Cucumber leaf samples in which CVYV was detected were also checked for the presence of CCYV and CYSDV by a DAS-ELISA using specific antisera against CYSDV, and CCYV (AS-0591, and AS-1020, respectively; DSMZ).

Mechanical transmission

Cucumber, melon, and squash plants were grown in a temperature controlled greenhouse at 25°C with a photoperiod of 14 h of light, kept in insect-proof cages, and used as test plants. For mechanical inoculation, the sap was extracted 1:1 (W/V) in 0.1 M phosphate buffer (pH 7.2) from leaf samples in which CVYV was detected by TAS-ELISA, and rubbed onto five test plants at the 2–4 leaf stage. The inoculated plants were placed in an insect-proof cage for symptom observation. The inoculated plants, were tested for CVYV, CCYV, and CYSDV infections 28–30 d post inoculation (dpi) using DAS/TAS ELISA.

Whitefly transmission

Groups of 20–25 viruliferous whiteflies (*B. tabaci*) were collected from CVYV infected greenhouse cucumbers and put on uninfected cucumber seedlings at the 2–6 leaf stage, giving a 72 h inoculation access period (IAP). Plants were kept in an insect proof cage. After the IAP, the seedlings were sprayed with Metasystox-R (EC 25%) insecticide (Oxydemeton-methyl, Bayer CropScience), and further cultivated in an insect-proof cage to study symptom development. The whitefly inoculated plants were also tested for the whitefly transmissible cucurbit infecting viruses CCYV and CYSDV using DAS-ELISA.

Figure 1. Severe vein clearing symptom in greenhouse-grown cucumber plants naturally infected with *Cucumber vein yellowing virus* in Tehran (Varamin), Iran.

Figure 2. Map showing the provinces in Iran that were surveyed for *Cucumber vein yellowing virus* during 2011–2012: 1, Semnan; 2, Tehran; 3, Isfahan; 4, Yazd; 5, Kerman; 6, Fars, 7; Hormozgan; and 8, Bushehr.

Amplification and sequence analysis

Total RNAs were extracted from 50 mg leaf samples using the RNeasy Plant Mini Kit (Qiagen) following manufacturer's instructions. Amplification was performed using the one-step RT-PCR kit (Invitrogen) using primers CVYV302-s2_(5'-CCAAAGCTCAGCAAAGAGAGG-3') and CVYV304-as2 (5'- GCATTGGGTTGTCCACAAG-3') which were designed to amplify a 503 bp fragment covering the C terminal part of the nuclear inclusion protein b and N terminal part of the viral coat protein. The following cycling conditions were used: reverse transcription for 30 min at 45°C, activation of polymerase for 2 min at 94°C followed by 34 cycles of denaturation at 94°C for 20 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min. The amplified fragment of one sample was purified using the NucleoSpin Gel and PCR Extraction kit (Macherey-Nagel), ligated into

a pGEM-T vector (Promega), cloned (*E. coli* DH5alpha, Amersham Pharmacia) and sequenced. The sequence analyses were performed using EMBL-EBI ClustalW2 server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MEGA5 (Tamura *et al*., 2011).

Results

TAS-ELISA

The ELISA results showed that 166 out of 394 cucumber leaf samples originating from cucumber greenhouses were positive for CVYV (Table 1), confirming the presence of CVYV in all surveyed provinces, except Bushehr. In addition, 107 leaf samples were collected from melon, squash, and cucumber, grown in open fields of Semnan, Bushehr, and Tehran provinces. When analyzed by ELISA, 15 were infected with CVYV, 49 with CCYV and 30 withCYSDV.. CVYV was not found in open-fields of Bushehr province.

Of all investigated samples, double or triple infections with CCYV and CYSDV occurred in 49 out of 166 of the CVYV-infected samples. CVYV+CCYV (29/49) was the most frequent double infection, followed by CVYV+CYSDV (6/49). The CVYV and CCYV combination was more prevalent than the CVYV and CYSDV combination. Triple infections by CVYV+CCYV+CYSDV were found in 14 out of the 49 samples.

Table 1. Number of greenhouse grown cucumber samples that were tested positive by ELISA for the presence of *Cucumber vein yellowing virus* (CVYV) from a survey undertaken in eight provinces of Iran during 2011–2012.

Province	Number of samples tested	Number of CVYV infected samples (%)
1- Tehran	90	26 (28.9%)
2- Semnan	40	27 (67.5%)
3- Hormozgan	27	22 (81.5%)
4- Yazd	55	25 (45.5%)
5- Esfahan	102	42 (41.2%)
6- Bushehr	8	0 (0%)
7- Fars	25	17 (68%)
8- Kerman	47	7 (14.9%)
Total	394	166 (42.1%)

Mechanical transmission

Vein clearing symptoms were observed on ten out of 15 mechanically inoculated cucumber plants 21–28 dpi. The presence of CVYV in six inoculated plants was confirmed by TAS-ELISA. CYSDV and CCYV were not detected in mechanically inoculated plants.

Whitefly transmission

Mild vein clearing symptoms were evident in four out of ten whitefly inoculated plants 25–30 dpi. The presence of CVYV in these plants was confirmed by TAS-ELISA and RT-PCR. CYSDV and CCYV were not detected by ELISA in any whitefly inoculated plant.

Sequence analysis

The 503 bp nucleotide sequence of the C terminal part of the nuclear inclusion protein b and N terminal part of the viral coat protein of the CVYV isolate obtained in this study has been deposited under GenBank accession no. KF482073. This sequence was compared with other CVYV sequences available at GenBank which cover the same region of the genome. The Iranian CVYV isolate showed the greatest nucleotide sequence identity to Spanish CVYV isolates (99.4–99.6%), followed by 95.1–95.5% sequence identity to isolates from Jordan (Figure 3). The aa sequence identities ranged from 96.4–97.6% with Jordan isolates and up to 99.4–100% with the isolates from Spain.

Discussion

In recent decades, several whitefly-transmitted viruses have been reported worldwide causing yellowing diseases in glasshouse- and open field-grown cucurbits (Wisler *et al*., 1998). During a recent survey in Iran, CYSDV and CCYV were reported in 49% and 19% of greenhouse grown cucumbers, respectively (Bananej *et al*., 2013), but the incidence of CVYV was not determined.

Here, the incidence of CVYV was also studied. TAS-ELISA results showed that 42% (166/394) from all col-

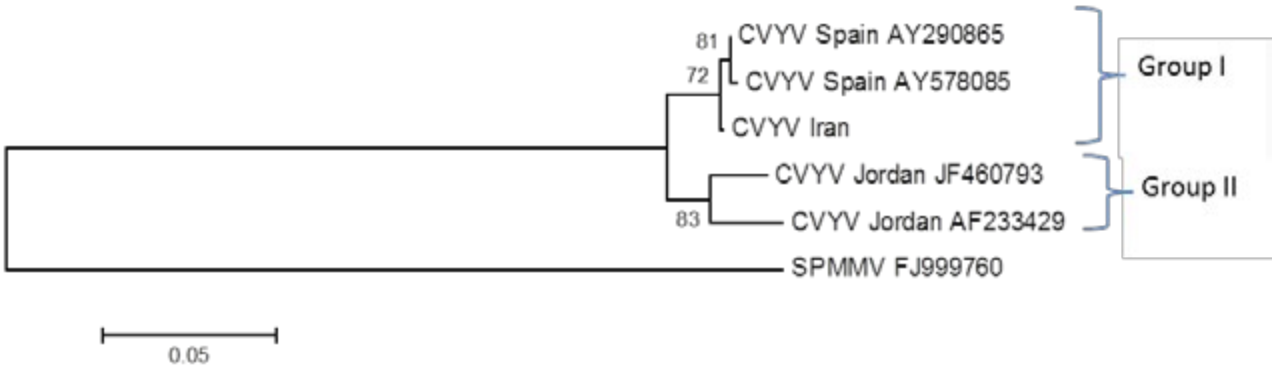

Figure 3. Phylogenetic tree based on *Cucumber vein yellowing virus* (CVYV) sequences available at GenBank and the isolate from Iran. The tree was inferred with 1000 bootstrap replicates of the maximum likelihood procedure applying MEGA 5 default settings. Bootstrap values above 70% are shown at the nodes. *Sweet potato mild mottle virus* (SPMMV) was used as the out group.

lected samples were positive; CVYV was found in all surveyed provinces, except Bushehr. CVYV was also detected in 15 out of 107 (14%) samples that where collected from field-grown melon and cucumber in Semnan and Tehran provinces, in the north of Iran. Furthermore, double or triple infections with CCYV and CYSDV occurred in 30% of the CVYV-infected samples. The CVYV and CCYV combination (59%) was more prevalent than the CVYV and CYSDV combination (12%). Triple infections (CVYV+CYSDV+CCYV) were found in 29% of the mixed infected samples.

CVYV was not found in open-fields of Bushehr province. It is possible that the virus has not yet been introduced into the Bushehr province, or that a larger number of samples need to be tested to verify its occurrence. The highest rate of infection was observed in Hormozgan and Fars provinces in the south of Iran and Semnan province in the north of the country. During a survey conducted in July 2002, CVYV was only reported from a cucumber plant (field-grown) in one region near Jiroft (Kerman Province) in the south of Iran (Bananej *et al*., 2006). Since 2002, no data are available on the status of CVYV in the major cucurbit growing regions of Iran. Based on the results obtained in this study, it is likely that CVYV was present in the cucurbit growing regions for a long time, but remained unknown because symptoms were mistaken to be caused by other common cucurbit infecting viruses or nutritional or physiological disorders. The results obtained in this study clarify the status of CVYV infections in the major cucurbit growing regions of Iran.

In this study, typical vein yellowing symptoms induced on whitefly (*B. tabaci*) and mechanically inoculated cucumber plants grown under greenhouse conditions indicate the role of CVYV in the vein yellowing disease which is observed in many naturally infected cucumber greenhouses in Iran. The role of the CVYV as a causal agent of vein yellowing symptoms found in Iranian glasshouses was confirmed by TAS-ELISA, RT-PCR and sequencing. Some whitefly–transmitted viruses belonging to the family *Closteroviridae* (genus *Crinivirus*) and aphid-transmitted viruses of the family *Luteoviridae* (genus *Polerovirus*) can also infect cucumber and induce yellowing symptoms, but in contrast to CVYV, they are not mechanically transmissible.

The partial nucleotide sequence of one Iranian CVYV isolate was determined and compared with previously characterized CVYV isolates for which sequences are available at GenBank. The nucleotide sequence identities to other CVYV isolates ranged from 95.1 to 99.6%. Phylogenetic analysis showed that isolates can be divided into two phylogenetic groups (I and II) (Figure 3). Despite of the close proximity of Jordan to Iran, the Iranian CVYV isolate clustered with Spanish isolates in group I and not with the Jordanian isolates of group II. However, more sequences of other isolates and further genetic studies are needed to identify the origin of CVYV in Iran.

In recent years cucumber cultivation in greenhouses has developed in many regions of Iran, and simultaneously, yellowing symptoms likely to be associated with viruses were frequently found in many

provinces. The sources of inoculum and immigration of vector populations are the main factors which drive the introduction and spread of viruses in general including CVYV. Epidemics of disease caused by CVYV could be affected by the number of whiteflies and mean maximum temperatures in greenhouses during the first crop month (Ruiz *et al*., 2006). In countries of the Middle East, diseases similar to that caused by CVYV have been observed mostly during the hottest cropping seasons, when the greatest numbers of whiteflies are produced (Cohen and Nitzany, 1960; Ucko *et al*., 1998**)**. The indoor climate may interact with virus–vector or virus–plant relationships and affect the seasonal incidence of whitefly-transmitted diseases in greenhouses (Harrison, 1981). The key to the management of whitefly transmitted viruses in greenhouse cucumber production lies in restricting the entrance and indoor mobility of the vector (Ruiz *et al*., 2006).

During recent years, the use of *Bemisia*-free seedlings, insect-proof greenhouses and removal of crop residues have been recommended in Iran. *Bemisia tabaci* populations are difficult to control with pesticide treatments due to insecticide resistant populations. Biological control of *B. tabaci* has been attempted in different cucurbit growing regions in Iran in recent years, but its impact on whitefly transmitted viruses, specifically on CVYV epidemics, has not yet been investigated. Further studies are required to evaluate the potential of these approaches to reduce the spread of these viruses and to preserve productivity in cucurbits.

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