OCCURRENCE AND DISTRIBUTION OF TEN VIRUSES INFECTING CUCURBIT PLANTS IN GUILAN PROVINCE, IRAN

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Summary. – During the 2006 and 2007 growing seasons, a systematic survey was conducted in open-field of melon (*Cucumis melo* L.), cucumber (*C. sativus* L.), squash (*Cucurbita* sp.), and watermelon (*Citrulus lanatus* L.) crops in 16 major cucurbit-growing areas of Guilan province in Iran. Symptomatic leaf samples were collected and screened by double-antibody sandwich ELISA (DAS-ELISA) or RT-PCR to detect Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus (WMV), Cucurbit aphid-borne yellows virus (CABYV), Cucumber mosaic virus (CMV), Squash mosaic virus (SqMV), Papaya ringspot virus type W (PRSV-W), Watermelon chlorotic stunt virus (WmCSV), Melon necrotic spot virus (MNSV), Zucchini yellow fleck virus (ZYFV), and Ourmia melon virus (OuMV). The majority of tested samples (73.7%) were infected by at least one of the viruses considered. OuMV, ZYMV, WMV, and WmCSV were the most prevalent viruses and were detected in tested cucurbit plants. The incidence of multiple infections with 2 or more viruses was also relatively high, 63.3, 48.6, 42.7, and 26.7% of the infected samples of melon, cucumber, squash, and watermelon, respectively. The high incidence of OuMV and WmCSV suggested that these viruses might turn out to be an important threat for the melon and cucumber crops in the province.

Key words: cucurbit viruses; incidence; DAS-ELISA; RT-PCR; multiple infection

Introduction

Cucurbit viruses have always been a major cause of quantity and quality reduction in cucurbit crops worldwide. Several viral diseases are responsible for significant economic losses in commercial cucurbit production. More than 35 viruses have been isolated from cucurbits (Provvidenti, 1996). ZYMV, WMV, CGMMV, WmCSV, CMV, CYSDV, PRSV-W, SqMV, OuMV, CABYV, and CVYV have been reported to infect field-grown melon, watermelon, squash, and cucumber in Iran (Bananej *et al*., 1998, 2002, 2006a,b; Keshavarz and Izadpanah, 2004; Parvizy, 1989; Ghorbani, 1988; Lisa *et al*., 1988; Izadpanah, 1978; Rahimian and Izadpanah, 1978; Ebrahim-Nesbat, 1974, 1972).

Melon, cucumber, squash, and watermelon are among the major vegetables grown in Iran, ranking first by economic value, second by production, and third by acreage. For example, Iran with 80,000 hectares is the third biggest producer of melon crop in the world after China and Turkey (F.A.O. 1996). The cucurbit crops are grown throughout Guilan province located in the northern part of the country (Fig. 1). Plants showing mosaic, leaf distortion, and yellowing

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Abbreviations: CABYV = Cucurbit aphid-borne yellows virus; CMV = Cucumber mosaic virus; CVYV = Cucumber vein yellowing virus; CYSDV = Cucumber yellow stunting disorder virus; DAS-ELISA = double-antibody sandwich ELISA; MNSV = Melon necrotic spot virus; OuMV = Ourmia melon virus; PRSV-W = Papaya ringspot virus type W; SqMV = Squash mosaic virus; WmCSV = Watermelon chlorotic stunt virus; WMV = Watermelon mosaic virus; ZYFV = Zucchini yellow fleck virus; ZYMV = Zucchini yellow mosaic virus

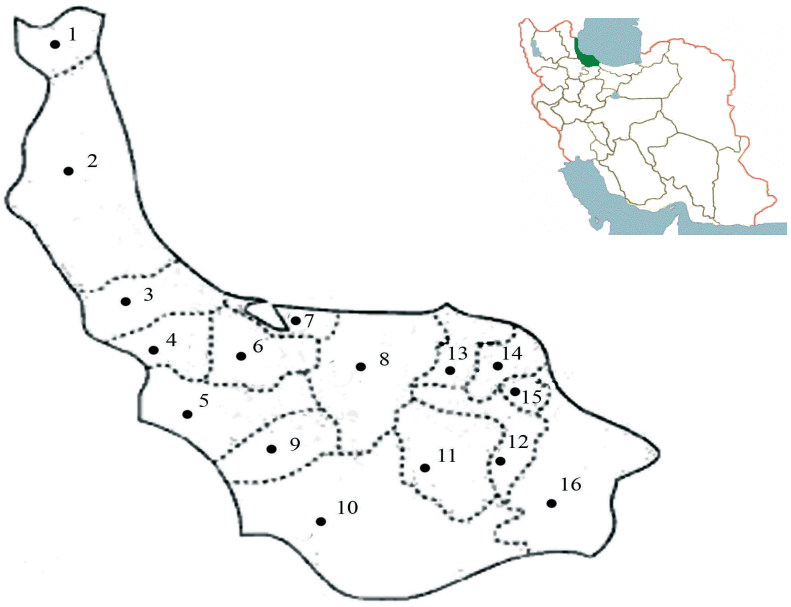

Fig. 1

Map of Guilan province showing the surveyed areas growing the cucurbits

1 – Astara, 2 – Talesh, 3 – Rezvanshahr, 4 – Masal, 5 – Fooman, 6 – Somesara, 7 – Bandar-e-Anzali, 8 – Rasht, 9 – Shaft, 10 – Roudbar, 11 – Siahkal, 12 – Amlash, 13 – Astaneh-Ashrafieh, 14 – Lahijan, 15 – Langeroud, and 16 – Roudsar.

symptoms were frequently found in the largest part of cucurbit fields. Despite the importance of field-grown cucurbits in the province, only limited information is available about the cucurbit viruses and the impact of viral disease on the production. About 11 different viruses were reported to infect *Cucurbitaceae* in Iran, but CMV and ZYMV have been reported only from Guilan province (Hamzeh *et al*., 2006; Danesh, 1969). However, in those studies a limited number of samples were tested and the distribution and relative incidence of the viruses detected was not determined.

In this study, we carried out a major survey of viral diseases during the growing seasons of 2006 and 2007 encompassing major regions of Guilan province, where cucurbit crops were field grown. We detected and estimated the incidence of infecting viruses and importance of viral diseases for cucurbit plants. The obtained data were essential regarding the search for control strategies of virus diseases in the cucurbit plants.

Materials and Methods

Survey and sample collection. During the period 2006 and 2007, a survey was conducted in 16 major cucurbit-growing areas of Guilan province in the north of Iran. A total of 590 leaf samples with virus-like symptoms were collected from melon (n = 119), squash (n = 150), cucumber (n = 159), and watermelon (n = 162) and tested for the presence of virus infection either serologically by DAS-ELISA or by RT-PCR.

To determine the plants where cucurbit viruses persisted during the host-free period, non-cucurbit samples (n = 129) and weed samples (n = 125) representing 18 species in 17 families (*Euphorbiaceae, Verbenaceae, Solanaceae, Polygonaceae, Papilionaceae, Convulvulaceae, Chenopodiaceae, Amaranthaceae, Rosaceae, Asteraceae, Apiaceae, Scrophulariaceae, Caryophyllaceae, Poacea, Plantaginaceae, Portulacaceae,* and *Lamiaceae)* were collected in or around cucurbit fields during the growing season or fields that grew cucurbit plants positive for cucurbit viruses during the host-free season.

Serology. The standard DAS-ELISA was performed according Clark and Adams 1977. IgGs and alkaline phosphatase conjugated IgGs for 10 viruses were used: CMV, CABYV, MNSV, PRSV-W, SqMV, WMV, ZYMV, WmCSV, ZYFV, and OuMV (kindly provided by Dr. H. Lecoq; INRA, Avignon, France). Each sample was tested in duplicates. The reaction was detected colorimetrically at $\lambda = 405$ nm using an ELISA reader (MCC-340, Multiscan Labsystem). Virus-free cucurbit species grown in insect-proof cages were used as negative controls. Also, positive controls were included in all tests. Samples were considered positive when the A_{405} value was 3 times higher than A_{405} of the healthy control.

RNA extraction. Total RNA was extracted from original leaves of ZYMV and CABYV-infected plants that were positive in DAS-ELISA using TRI-Reagent (Sigma). RNA resuspended in 20 µl H_2O was heated at 65°C for 5 mins before reverse transcription.

Table 1. Primers used in RT-PCR and predicted amplicon size for the detection of ZYMV and CABYV

Primer	Sequence	Amplicon size (bp)
ZYMV-CP-5'	5'-GGTTCATGTCCCACCAAGC-3'	600
ZYMV-CP-3'	5'-ATGTCGAGTATCACATTTCC-3'	
CABYV-CP-5'	5'-CGCGTGGTTGTGGTCAACCC-3'	479
CABYV-CP-3'	5'-CCYGCAACCGAGGAAGATCC-3'	

ZYMV RT-PCR. Four µl of the RNA were submitted to reverse transcription in a final volume of 20 µl using the ZYMV-CP-3' (5'-ATGTCGAGTATCACATTTCC-3', position 8756–8775 nt numbered according to AY188994), for 1 hr at 42°C with M-MuLV reverse transcriptase (Fermentas). 2.5 µl of the RT reaction mixture was used for PCR using the ZYMV-CP-5' (forward primer, position 8169–8187 nt), and ZYMV-CP-3' (reverse primer) (Table 1; Desbiez *et al*., 1996). PCR cycling condition were as follow: denaturation at 94°C for 3 mins, followed by 35 cycles at 94°C for 30 secs, 55°C for 30 secs, and 72°C for 30 secs, and a final elongation step at 72°C for 7 mins. PCR products were checked by an electrophoresis on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

CABYV RT-PCR. Four µl of the RNA were submitted to reverse transcription in a final volume of 20 µl, using the CABYV-CP-3' (5'-CCYGCAACCGAGGAAGATCC-3'), for 1 hr at 42°C with M-MuLV reverse transcriptase (Fermentas). 2.5 µl of the RT reactions were used for PCR using forward and reverse primers designed in conserved regions of the coat protein gene according to the sequence of a CABYV reference isolate (Table 1; Guilley *et al*., 1994). PCR cycling conditions and electrophoresis are described in previous section.

Results and Discussion

Detection, geographical distribution, and incidence of cucurbit viruses

Samples from 590 symptomatic plants collected from melon, cucumber, watermelon, and squash were analyzed using DAS-ELISA or RT-PCR to determine the presence of ZYMV, WMV, CABYV, CMV, SqMV, PRSV-W, WmCSV, MNSV, ZYFV, and OuMV viruses in 16 areas of Guilan province of Iran. 435 samples (73.7%) reacted positively in DAS-ELISA with at least one of the viruses examined. The percentage of infected samples taken from melon, squash, watermelon, and cucumber was relatively high 82, 64, 62, and 88%, respectively (Table 2). These results revealed that cucurbits grown in commercial fields were infected quite commonly with these viruses. Unfortunately, growers in the surveyed regions are not aware of the ways the viruses are spread from plant to plant and about precautions concerning the virus transmission. A significant percentage of samples (26%) from symptomatic cucurbit plants did not react with the antisera against any of the tested viruses, what may be due to the presence of non-identified viruses.

Table 2. Infection of the cucurbit samples by the examined viruses during the seasons 2006–2007

Cucurbit species	Infected/collected samples (%)	Non-identified/collected samples (%)
Melon	98/119 (82.4)	21/119 (17.6)
Squash	96/150 (64)	54/150 (36)
Watermelon	101/162 (62.3)	61/162 (37.7)
Cucumber	140/159 (88)	19/159 (12)
Total	435/590 (73.7)	155/590 (26.3)

RT-PCR experiments yielded an expected ~600 and ~479-bp product, similar to the fragment amplified with extracts from the reference isolates of CABYV and ZYMV (Desbiez *et al*., 1996; Guilley *et al*., 1994). No amplification of the product was observed from the healthy plant extracts (data not shown).

The incidence of the different viruses infecting cucumber, melon, squash, and watermelon is reported in Table 3. WMV, ZYMV, WmCSV, and OuMV were detected in 30.1, 29, 20, and 19.6% of the tested samples, respectively. OuMV, ZYMV, WMV, and WmCSV were detected in 58.8, 35.3, 45.6, and 50.9% of the melon, squash, watermelon, and cucumber samples, followed by WMV, WmCSV, ZYMV, and ZYMV, respectively (Table 3). CABYV, CMV, PRSV, ZYFV, MNSV and SqMV were in less than 8, 5, 3, 3, 2, and 1% of infected samples, respectively. WMV, ZYMV, WmCSV, OuMV, CMV, PRSV were detected in all four cucurbit species, but CABYV, ZYFV, MNSV, and SqMV were not detected in any sample taken from watermelon. These results showed that OuMV, ZYMV, WMV, and WmCSV were the most important and prevalent viruses in open field crops of the melon, squash, watermelon, and cucumber in Guilan province during 2006 to 2007, respectively. Of the viruses tested, ZYMV, WMV, and WmCSV were the most widespread viruses found in 75, 68, and 68% of the surveyed regions, respectively (Table 4).

OuMV has been reported for the first time from Azerbaijan-E-Gharbi in the west of Iran (Lisa *et al*., 1988). Since 1988, we have had no evidence about OuMV in other regions of Iran or other countries of the world. Results obtained in this study showed the occurrence of OuMV in the region of Iran previously considered as free of this virus. In agreement with our findings, WMV was the most prevalent virus in the Greece and Central Valley of California (Papavassiliou *et al*., 2002; Grafton-Cardwell *et al*., 1996). In Lebanon, ZYMV was the most common virus in zucchini (64%) and melon (44%) (Abou-Jawdah *et al*., 2000). In Pakistan, ZYMV and WMV are the prevalent cucurbit viruses (Ali *et al*., 2004).

In this study, all four cucurbit species in surveyed regions were infected with WMV, ZYMV, WmCSV, OuMV, CMV,

Table 3. Occurrence of examined viruses in four cucurbit plants grown in Guilan province

Plant	Collected sample	Infected samples (%)									
		WMV	ZYMV	WmCSV	OuMV	CABYV	CMV	PRSV	ZYFV	MNSV	SqMV
Melon	119	56(47)	18(15.1)	4(3.3)	70(58.8)	8(6.7)	12(10)	5(4.2)	4(3.3)	2(1.6)	0
Squash	150	20(13.3)	53(35.3)	24(16.0)	20(13.3)	20(13.3)	2(1.3)	1(0.6)	2(1.3)	2(1.3)	1(0.6)
Watermelon	162	74(45.6)	35(21.6)	9(5.5)	2(1.2)	0	4(2.4)	8(4.9)	0	0	0
Cucumber	159	28(17.6)	65(40.8)	81(50.9)	24(15)	17(10.6)	8(5.0)	1(0.6)	8(5.0)	4(2.5)	3(1.8)
Total	590	178(30.1)	171(28.9)	118(20.0)	116(19.6)	45(7.6)	26(4.4)	15(2.5)	14(2.3)	8(1.3)	4(0.6)

and PRSV. These results showed that cucurbit species cultivated in surveyed regions are susceptible to viral infection. An important alternative for the disease control would be the use of cultivars that carry a gene for a resistance to the infecting viruses.

Multiple infections

The number of multiple infections (double, triple, and quadruple) was found in approximately 45.5% (198/435) of the samples tested (Table 5). The positive samples were infected with two 33% (145/435), three 10 %(45/435), and four viruses 2 %(8/435), respectively. The number of multiple infections in melon (63%), squash (43%), watermelon (27%), and cucumber (49%) was relatively high (Table 5). In melon, squash, watermelon, and cucumber, the most common double infection was WMV/OuMV, ZYMV/OuMV, ZYMV/WMV, and ZYMV/WmCSV, followed by ZYMV-OuMV, ZYMV-WMV, WMV-WmCSV, and CABYV-WmCSV, respectively (Table 5). The most common triple infection was ZYMV/WMV/OuMV, ZYMV/CABYV/OuMV, ZYMV/WMV/WmCSV, and WMV/WmCSV/OuMV. Among the quadruple infections, the most frequent association was WMV-CMV-WmCSV-PRSV for watermelon and ZYMV-OuMV-WmCSV-WMV for cucumber (Table 5). In 1998 in Lebanon, 30% of the squash plants showed double infections with ZYMV/CABYV (Abou-Jawdah *et al*., 2000). The highest value (15%) was found for cucurbits of Turkey and Spain double infected with ZYMV/WMV (Sevik and Arli-Sokmen, 2003; Luis-Arteaga *et al*., 1998). Infection of plants by several viruses is a common phenomenon (Falk and Bruening, 1994). Double or multiple infections of plants often results in a higher severity of symptoms and virus accumulation, a phenomenon referred to as synergy (Matthews, 1991). Synergistic effect occurring for a number of virus combinations is probably related to the silencing suppression ability of the viruses involved (Hull, 2002). For viruses of the family *Luteoviridae*, the synergistic effects were described (Savenkov and Valkonen, 2001).

Table 4. Geographical distribution of viruses infecting cucurbit plants in 16 regions of Guilan province detected by DAS-ELISA or RT-PCR

Regions	WMV	ZYMV	WmCSV	OuMV	CABYV	CMV	PRSV	ZyFV	MNSV	SqMV
1-Astara	+	+	+	–	–	–	+	–	–	–
2-Talesh	+	+	+	–	–	–	–	–	–	–
3-Rezvanshahr	–	+	–	–	–	–	–	–	–	–
4-Masal	+	+	–	+	–	–	–	–	–	–
5-Fooman	–	–	–	–	–	–	–	–	–	–
6-Somesara	+	+	+	+	+	+	–	–	+	–
7-Bandar-e-Anzali	+	+	+	–	+	–	–	–	–	–
8-Rasht	+	+	+	+	+	+	+	+	+	+
9-Shaft	–	+	–	–	–	–	–	–	–	–
10-Roudbar	+	–	+	+	+	+	–	–	–	–
11-Siahkal	+	+	–	–	–	–	–	+	–	–
12-Amlash	–	–	–	+	–	–	+	–	–	–
13-Astaneh-Ashrafieh	+	+	+	–	–	+	+	+	–	+
14-Lahujan	–	–	+	–	–	–	–	–	–	–
15-Langeroud	+	+	+	–	–	+	–	–	–	–
16-Roudsar	+	+	+	–	+	–	–	+	–	–
Positive/surveyed regions	11/16	12/16	11/16	4/16	5/16	6/16	3/16	4/16	2/16	2/16

(+) = positive; (–) = negative.

Table 5. Multiple infections in cucurbits in the seasons 2005–2006

	Multiple infected/total samples/total of infected samples			
	Double	Triple	Quadruple	Total (%)
Squash	33/96 ZYMV-OuMV ZYMV-WMV	8/96 ZYMV-CABYV-OuMV ZYMV-WMV-CABYV	0/96 0	41/96(43)
Melon	43/98 WMV-OuMV ZYMZ-OuMV	19/98 ZYMV-WMV-OuMV CMV-WMV-OuMV	0/98 0	62/98(63)
Cucumber	45/140 ZYMV-WmCSV WmCSV-CABYV	16/140 WMV-WmCSV-OuMV ZYMV-WMV-CABYV	7/140 ZYMV-OuMV-WMV-WmCSV ZYMV-WMV-CABYV-WmCSV	68/140(49)
Watermalon	24/101 ZYMV-WMV WMV-WmCSV	2/101 ZYMV-WMV-WmCSV	1/101 WMV-CMV-WmCSV-PRSV	27/101(27)
Total	145/435(33)	45/435(10)	8/435(2)	198/435(45.5)

Detection of cucurbit viruses in non-cucurbit plants in the surveyed regions

To determine the plants serving for persistence of cucurbit viruses during the host-free period, the survey of the cucurbit production area was extended. DAS-ELISA results showed that cowpea (*Vigna unguiculata*) and pepper (*Capsicum annum*) were infected with PRSV, CMV, ZYFV, and WmCSV. PRSV and CMV was detected in common bean (*Phaseolus vulgaris* var. Contender). Soybean (*Glycine max*), pepper, and cowpea were infected with WmCSV, WMV, and CABYV, respectively (Table 6). To our knowledge, this is the first report of WmCSV infection detected in the cowpea, soybean, and pepper.

Some weed species served as alternate hosts for plant viruses. A total of weed samples (n = 125) were examined and ZYFV, CMV, PRSV, WmCSV, and MNSV were detected in 5 genera of weed samples represented by *Solanum nigrum (Solanaceae*), *Amaranthus* sp. (*Amaranthaceae*), *Portulaca oleracea* (*Portulacaceae*), *Xanthium strmarium* (*Asteraceae*), and *Chenopodium album* (*Chenopodiaceae*). PRSV was detected in *S. nigrum*, *Amaranthus* sp., *P. oleracea*, and *X. strmarium*. CMV was detected in *S. nigrum* and *Amaranthus* sp. MNSV and WmCSV were detected in *S. nigrum* and *Ch. album*, respectively (data not shown). Our results suggested that these plant species were important as presumable alternate hosts to the cucurbit viruses. Certainly, this suggestion is remarkable, but the survey lasting only for

Table 6. Detection of the examined viruses in non-cucurbit plants in the surveyed regions

Non-cucurbit plants	Collected samples	No. of positive samples									
		PRSV	CMV	ZYFV	WmCSV	WMV	CABYV	ZYMV	MNSV	SqMV	OuMV
Papilionaceae											
Vigna unguiculata	32	7	1	4	1	0	2	0	0	0	0
Phaseolus vulgaris cv. Top-crop	30	0	0	0	0	0	0	0	0	0	0
Phaseolus vulgaris cv. Contender	16	1	2	0	0	0	0	0	0	0	0
Glycine max	6	0	0	0	1	0	0	0	0	0	0
Arachis hypogaea	11	0	0	0	0	0	0	0	0	0	0
Vicia faba	1	0	0	0	0	0	0	0	0	0	0
Solanaceae											
Solanum melongena	2	0	0	0	0	0	0	0	0	0	0
Capsicum annuum	25	1	4	1	1	2	0	0	0	0	0
Nicotiana glutinosa	1	0	0	0	0	0	0	0	0	0	0
Brassicaceae											
Raphanus sativus	5	0	0	0	0	0	0	0	0	0	0
Total	129	9	7	5	3	2	2	0	0	0	0

2 years is not sufficient enough for definitive conclusions. We feel that a further thoroughgoing survey is required.

Summing up, the high incidence of single infection with OuMV and WmCSV in melon and cucumber samples (59% and 51%, respectively), double infections with WMV-OuMV in melon and ZYMV-WmCSV in cucumber and the first-time detection of OuMV in the new regions of Iran suggested that OuMV and WmCSV might become an significant threat for melon and cucumber crops in Guilan province and a probable treat for cucurbit crops in the future. This is the first report of an extensive survey carried out using serological and molecular diagnostic procedures to determine the incidence of most important viruses of the cucurbit crops in Guilan province.

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