Occurrence, Distribution and Biological variability of Zucchini Yellow Mosaic Virus in cucurbits of Khuzestan province, South west of Iran

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Abstract

ZYMV is one of the most important plant viruses that cause economical damage in cucurbits. The symptoms of ZYMV in different cucurbits include stunting, yellowing, mottling, severe mosaic, leaf and fruit deformation, blistering and shoe string. Investigation on occurrence of this virus, in Khuzestan province was carried out in November 2009, April and May 2010 by collecting cucurbits samples from different cucurbits fields. After DAS-ELISA test, ZYMV was maintained in squash. Then total RNA were extracted and were tested by RT-PCR. Using RT-PCR, fragments belonging to N-terminal of coat protein and C-terminal of nuclear inclusion bodies were replicated. PCR product for investigation of replication was loaded in 1% agarose gel. From seven regions in Khuzestan, 175 leaf samples showing different symptoms (yellowing, mosaic, deformation and blistering) were collected. Seventy one samples out of total samples (175 samples) showed ZYMV infection. Occurrence of Zucchini Yellow Mosaic Virus in Khuzestan province was confirmed, using serological and RT-PCR tests. Infection of ZYMV in Khuzestan province (40.5%) is higher than the average of Iran's infection (38%). This article is first report of occurrence ZYMV in different regions of Khuzestan province except Dezful.

Introduction

Virus diseases are a worldwide problem of cucurbits and a major limiting factor for cucurbits cultivation. About 35 viruses infecting cucurbits has been known worldwide.[1] Zucchini Yellow Mosaic Virus (ZYMV, *Potyvirus*) is one of the most damaging *emerging* viruses of cucurbits.[2] ZYMV was first isolated in 1973 and described by Lisa *et al.* in 1981[3] is the cause of one of the most economically important diseases of the family Cucurbitaceae, naturally infecting plants in more than 50 countries.[4] The virus was detected for the first time in Iran in 1988, by Ghorbani.[5,6] ZYMV belong to the *Potyvirus* genus and *Potyviridae* family. The virus also called as Muskmelon Yellow Stunt Virus.[7] The symptoms of ZYMV in different cucurbits include stunting, yellowing, mottling, severe mosaic, leaf and fruit deformation, blistering and shoe string.[8] ZYMV infects cucurbit plants and is transmitted non-persistently by many colonizing and non-colonizing aphid species. In this mode of transmission aphids transmit the viruses by short test probes, lasting a few seconds, to evaluate the suitability of the host plant during the host selection process. To date, 10 aphid species of the family Aphididae have been reported as vectors of ZYMV.[9] Iran with 6935000 tones cucurbits production is the third major producer of this crop in the world, after China and Turkey.[10] Out of total cucurbits production in Iran, 5.05% produce in Khuzestan province. Thus this province with more than 29 tones cucurbit production is sixth major producer of cucurbits in Iran.[13] More than 24 tones cucurbits were exported from Khuzestan province to Iraq and neighboring countries in Persian Gulf.[14] More recently, symptoms of viral diseases similar to ZYMV, which caused heavy damage to cucurbits, have been reported from Khuzestan province. In this study, we investigated occurrence and distribution of ZYMV in cucurbit crop in Khuzestan province.

Materials and Methods

Sample Collection

Samples were collected from seven main cucurbits cultivation regions of Khuzestan province namely Sush, Shushtar, Molasani, Hamidieh, Dezful, Dashte Azadegan, Behbahan. Sampling was carried out on November 2009, April and May 2010 by collecting cucurbit leaves samples from different cucurbits fields. These regions are located in north, central, west and south east of Khuzestan province (Figure 1). Other regions of Khuzestan province haven't cucurbit's fields or their cultivations were very low. From each region, three fields were selected and 8-9 samples were collected from each field randomly. Moving in each field was in W-form. In all, 25 samples were collected from each region.

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Key words: Zucchini Yellow Mosaic Virus, DAS-ELISA, Distribution, Khuzestan province, RT-PCR.

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Serological assay

Samples were tested by DAS-ELISA using Clark and Adams' method.[15] Serological test was performed by polyclonal anti serum and positive control (Bioreba, Swiss) kindly provided by Dr C. Powell (IRREC, University of Florida, USA). ELISA Plate was pre-coated with coating antibody that was diluted in carbonate buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 per L, pH 9.6) at a ratio 1:1000, and incubated for 2-4 h at 37°C. The plate was washed with PBST buffer (8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4.12H2O, 0.2 g KCl, 0.2 g NaN_3, 0.5 mL Tween 20 per L, pH 7.4) three times, each time for 3 min. Leaf samples were grounded in mortar and pestle with an extraction buffer (PBST+2% PVP, pH 7.4) at a ratio 1:10 and were placed in wells. Plate was incubated at 4°C overnight and washed three times with PBST-Tween 20 buffer. Then Plate was coated with alkaline phosphatase conjugated antibody diluted in conjugate buffer (PBST, 2% PVP (polyvniylpyrrolidone), 0.2% egg albumin, pH 7.4) at a ratio 1:1000, and incubated for 2-4 h at 37°C. After washing, 10 mg p-nitrophenyl phosphate in substrate buffer (97 mL diethanolamine, 0.2 g NaN_3, pH 9.8) was added to each well and incubated at room temperature for 120 min. The reaction was detected at OD_{405nm} using ELISA reader (MR5000, Dynatech, USA). Two wells were used per sample. Virus-free cucurbit species grown in insect-proof cages were used as negative con-

trols. Samples were considered to be positive if the $OD_{405\ nm}$ values were more than two times the average healthy control.

Survey virus in greenhouse and host range

Positive samples were maintained in squash seedling (*Cucurbita pepo* cv. White Bush) with potassium phosphate buffer 0.01M using carburandum dust. For biological isolation, sap of infected leaf was inoculated to *Chenopodium quinoa*. Each local-lesion of *Chenopodium quinoa* leaf was passed 3 times. Three plants of each species were inoculated to each isolates. In addition, 13 species of 5 families inoculated and symptoms were investigated for one month.

Extraction of total RNA

Total RNA were extracted with phenol-chloroform method. For this procedure, 0.1 g fresh squash leaf was grounded by liquid nitrogen. Then 200 µL extraction buffer containing glycine 0.1M, NaCl 0.1 M, EDTA 0.01M, 2-mercaptoethanol 1% and 1% SDS, pH=9 was added. Then 300 µL phenol (equal volume of buffer) was added and inverted microtube slowly. Chloroform half volume of phenol (150 µL) and 1:25 volume of phenol (6 µL) isoamyl alcohol was added and the microtube was inverted slowly. Microtube was kept in freezer for 5 min. Then microtube was centrifuged 4 min at 13000 rpm and 4°C. Supernatant was transformed to another sterile microtube. If supernatant was not clear, this step was repeated and phenol-chloroform- isoamyl alcohol was added again. When supernatant was clear, 1.5 volume of supernatant ethanol and 0.1 volume of supernatant sodium acetate 3M, pH=5 were added. The microtube was kept in freezer overnight. Later on, this microtube was centrifuged at 13000 rpm for 16 min at 4°C. Then supernatant was removed and pellet was washed with ethanol 70%. After removing ethanol, pellet resolved in 20 µL RNase-free water. The quantity of RNA (A260/280) was measured with Nanodrop1000 (Termo scientific, USA).

Reverse transcription polymerase chain reaction

For RT-PCR, reverse primer(R-DAG) with this sequence GCGTGGCAATGACAT which is located in 8735-8749 of genome of ZYMV. RT reaction was performed in 20 µL total volume containing 2 µL 5X RT buffer, 1 µL DTT 0.1M, 1 µL dNTPs 10 mM, 1 µL R-DAG primer (10 pmol/µL), 0.5 µL RNase inhibitor(40 u), 0.5 µL MMuLV-RT(200 u) and 3 µL RNA. RT reaction was performed in 1cycle with 2 steps: 72°C (3 min) and 42°C (40 min). After RT reaction, PCR was performed using forward primer (F-DAG) having sequence ATTTGCGCTGCGATG that is located in 8291-8305 of genome. Record number of primers in gene bank is L31350.[18] 5 µL CDNA, 5 µL 10X PCR buffer, 2 µL $MgCl_2$, 1µL dNTPs(10 mM), 1 µL R-DAG primer (10 pmol/µL), 1 µL F-DAG primer (10 pmol/µL), 0.5 µL Taq polymerase(5 u) was used in PCR reaction. PCR reaction was performed in 50 µL total volume by first denaturation at 94°C for 3 min and followed by 35cycles with this program: 94°C for 30s, 43°C for 30s, 72°C for 30s and final elongation step at 72°C for 7 min. Then RT-PCR products were loaded in 1% agarose gel.

Results

Sample collection and serological assay

From seven regions in Khuzestan, 175 leaf

Figure 1. Geographical location of seven sampling regions in Khuzestan province.

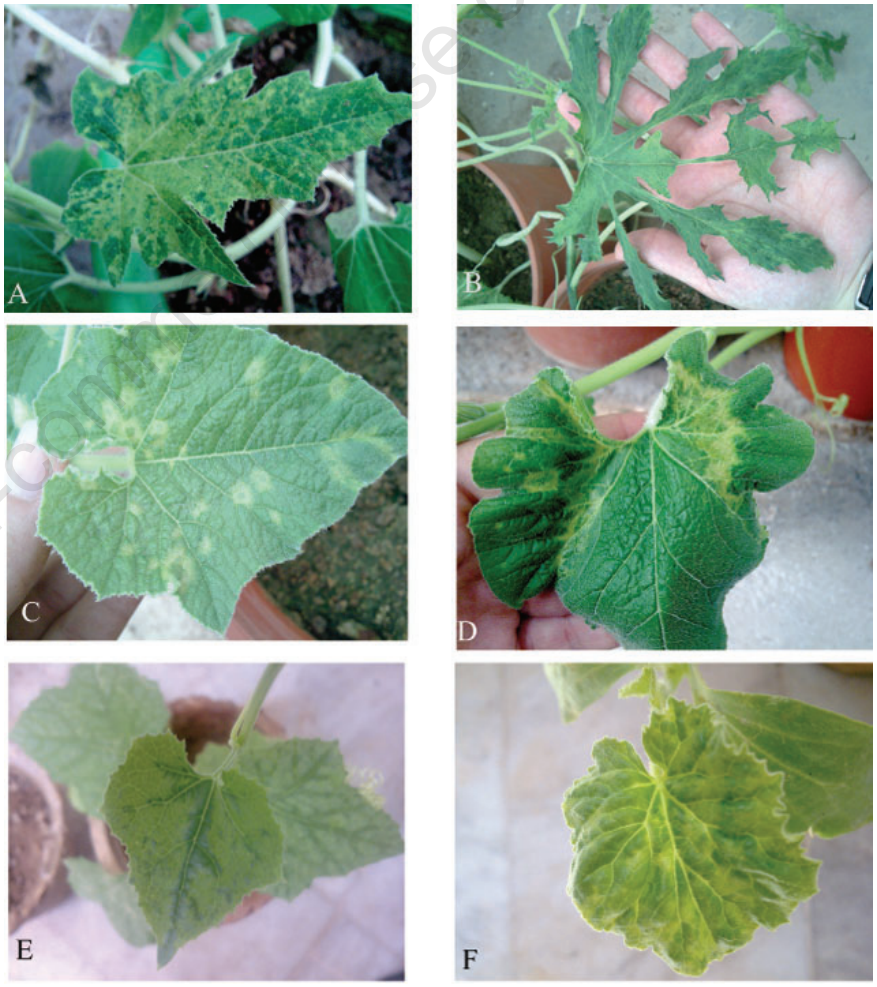

Figure 2. Symptoms of Khuzestan isolates. Mosaic & leaf deformation in *Cucurbita pepo* cv. White Bush by Dezful isolate (A). Leaf deformation & shoe stringing caused by Shushtar isolate (B). Ring spot in *Cucurbita pepo* cv. Sefide Gavi caused by Hamidieh isolate (C). Rugose & mosaic caused in *Cucurbita pepo* cv. Sefide Gavi by Molasani isolate (D). Green veining banding in *Luffa acutangula*, 2 week after inoculation (E). Blistering & mosaic in *Cucumis melo* cv. Abas Shori (F).

samples showing different symptoms (yellowing, mosaic, deformation and blistering) were collected. Samples were obtained from 21 cucurbit's fields.

Seventy one samples out of total samples (175 samples) showed ZYMV infection. Table 1 reveals percentage of infection from each region. In Dezful (68%), ZYMV infection was higher than other regions and Shush (8%) was lowest. In squash, ZYMV infection was higher than other cucurbit crops (Table 2).

Symptomology and host range

Seven isolates were inoculated to 13 species. These isolates caused different symptoms (Table 3). Symptoms include yellowing, stunting, vein clearing, mosaic, blistering in leaf and severe leaf malformation that later on became as shoe stringing leaves. Investigation on symptoms of isolates showed that Dezful, Shush, Shushtar, Hamidieh and Molasani caused Ring sopt in *Cucurbita pepo* cv. Sefide Gavi.

Dashte Azadegan isolate was only isolate

Table 1. Name of region and percentage of infection in each region.

Region	Percentage of infection
Shush	8%
Molasani	48%
Hamidieh	28%
Shushtar	52%
Dashte Azadegan	28%
Dezful	68%
Behbahan	52%
Total	40.5%

Table 2. Percentage of infection of ZYMV on different cucurbit crops in Khuzestan province.

Crops	Total number ample of each crop	Total number of infected samples from each crop	Percentage of infection
Cucumber	42	11	26.1%
Squash	34	29	85.2%
Snake cucumber	42	13	30.9%
Water melon	32	8	25%
Melon	25	10	40%
Total	175	71	40.5%

Table 3. Host range of seven isolates of Khuzestan province.

Test plants isolaates	Shush	Shushtar	Hamidieh	Molasani	Dezful	Dashte azadegan	Behbahan
Cucurbitaceae							
Cucurbita pepo cv. white Bush	St, M, Mo,Y	Sm, B, Ml,Ss	Sm, Mo,Ml	M,Ml, Mo, Ss	Sm, Vc, Ss,Ml	M,Vc, Ml,B	Mm, Mo, Ml
Cucurbita pepo cv. Sefid Gavi	Rs, Gv, Y, Mo, Mm	Sm, Mo, Rs	M,Mo,Ml,Vc,Rs	M,Rs, Mo,Gv	Sm,Mo, Rs	Sm, Mo, Gv	Y, Vc
Citrullus lanatus cv. Crimson Sweet	Mm, Y	M	Mm, Y	M, Y	M, Y	Mm, Y	Mm,Y
Cucumis sativus cv. Prince	M	M, Vc	M, Vc	M, Y	M	M	M
Cucumis sativus cv. Super Dominus	M	M	M	M	M, Mo	M	M
Cucumis melo cv. Mashhadi	M	M,B	M,Y	M,B	M, B	M,B	M, B
Cucumis melo cv. Abas Shori	M,B	M,Y,B	M,B	M, B, Mo, Gv	M, B	M, B	M, B
Luffa acutangula	Mm	Mm, Y,Gv	M	M, Gv	Mm	Mm	Mm
Chenopodiacae							
Chenopodium quinoa	Cll	Cll	Cll	Cll	Cll	Cll	Cll
Chenopodium amaranticolor	Cll	Cll	Cll	Cll	Cll	Cll	Cll
Amaranthaceae							
Gomphrena globosa	Nll	Nll	Nll	Nll	Nll	Nll	Nll
Solanaceae							
Datura metel	No	No	No	No	No	No	No
Fabacae							
Vigna unguiculata	Mm	Mm	Mm	Mm	Mm	Mm	Mm

Mo, mottling; M: Mosaic, Y, yellowing; Sm, severe mosaic; Mm, mild mosaic, Ss, shoe stringing; Vc, vien clearing; Gv, green veining banding; B, blistering; St, stunting; Rs, ring spot R, rugose; Ml, malformation; Cll, clorotic local lesion; Nll, necrotic local lesion; No, no symptom.

that caused blistering in *Cucurbita pepo* cv. White Bush. Behbahan isolates were caused mild mosaic in *Cucurbita pepo* cv. White bush (Figure 2).

Extraction RNA & RT-PCR test

With phenol- chloroform, total RNA were extracted. The range of $A_{260/280}$ RNA was 1.8-2. With special primers of ZYMV, seven isolates that collected from several region of Khuzestan were replicated. The fragments with 458 bp length were amplified in each isolates. Bands of isolates revealed in 1% agarose gel (Figure 3).

Discussion

According to the Iranian Agricultural Statistics of years 2005-2006, 2006-2007, 2007-2008, Khuzestan province has dropped from second place producing cucurbits region in Iran to sixth place.[11-13] Drought and various diseases including viral diseases of cucurbits may have been the cause. Among the viral diseases of cucurbits, ZYMV is the most destructive viral diseases in many parts of the world.

This virus causes severe damage to cucurbits crop and reduction of marketable fruits harvested from infected fields.

Ulman *et al.* and Krstic *et al*. studied in Hawaii and Serbia showed that the main viral disease in Hawaiian and Serbian cucurbit crops is ZYMV.[16,17] In the present study, investigation on presence of this virus in cucurbit fields of Khuzestan has been carried out in seven major regions of cucurbit's cultivation namely Molasani, Hamidieh, Shushtar, Sush, Dezful, Dashte Azadegan and Behbahan, in total 175 samples were collected.

After DAS-ELISA test, this virus was detected in 71 samples. To confirm DAS-ELISA results and relative serological between ZYMV and WMV, we performed RT-PCR test with specific primers of ZYMV.

RT-PCR test results and review of bands on 1% agarose gel also indicated presence of the virus in Khuzestan province. This result with the results of Desbeiz *et al.* and Hosseini *et al.* is consistent.[18,19] Symptomology studies of Khuzestan isolates indicated wide range of symptoms including yellowing, stunting, vein clearing, mosaic leaf, severe leaf malformation and shoe stringing leaves. Symptoms on squash cultivar White Bush caused by Sush and Behbahan isolates were different from other isolates. Behbahan isolate caused only mild mosaic and Shush isolate caused severe stunting, mild mosaic and vein clearing. Dashte Azadegan and Shushtar isolates were only isolates that caused blistering in squash cultivar White Bush. In squah cultivar Sefide Gavi, Molasani, Shoshtar and Dezful caused ring spot whereas other isolates caused severe/ mild mosaic, mottling, yellowing and vein clearing. All isolates caused symptom in *Vigna unguiculata* but didn't cause any symptoms in *Datura metel*. In all, Dezful, Shushtar and Molasani Isolates caused severe symptoms more than other isolates and Behbahan isolate caused symptoms less than others. Determination of the distribution of ZYMV infection in Khuzestan province indicated Dezful with 68% infection had highest rates of infection among the other regions. The reason may be attributed to continuous cultivation of cucurbits in this region, high cultivation squash (preferred host of ZYMV) & high aggregation and efficiency of vectors. Shush had lowest infection rate. According to observation symptoms similar to ZYMV (Blister and mosaic in fruit & mosaic in leaf) in cucumber fields of Shush, It's possible that CMV also were spread in this region. Infection of ZYMV in Khuzestan province (40.5%) is higher than the average of Iran's infection (38%).[20] Among cucurbit crops in Khuzestan province, squash had highest percent of ZYMV infection (85%) and after the Squash, Melon (40%), Snake cucumber (30.9%), Cucumber (26.1%) and Watermelon (25%).

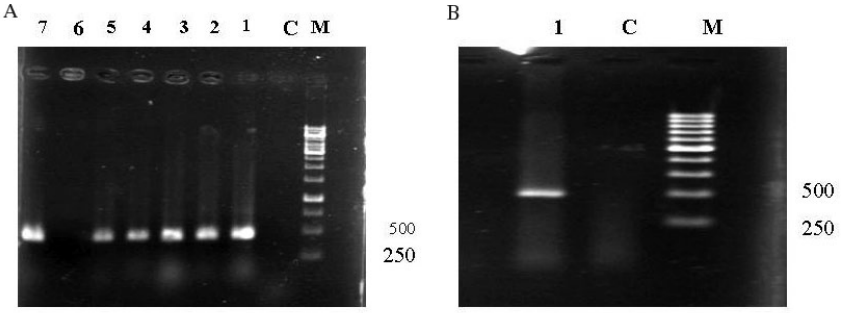

Figure 3. Bands of RT-PCR products of Khuzestan isolates. M: marker 1kb (Gene Ruler™ 1Kb, Fermentas) & C:Negative control. (A): 1-Molasani, 2- Hamidieh, 3- Shushtar, 4- Shush, 5- Dezful, 6- whole well, 7- Dashte Azadegan (B):1-Behbahan.

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