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Characterization of the *Zucchini Yellow Mosaic Virus* from Squash in Tehran Province

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ABSTRACT

***Zucchini yellow mosaic virus* (ZYMV) is a potyvirus with a worldwide distribution. This virus causes serious economic losses in Iran in many cucurbits. During 2002-2003, samples were collected from squash fields in Tehran Province. Five isolates (Z1, Z2, Z3, Z4 and Z5) were inoculated on 27 species of Cucurbitaceae, Chenopodiaceae, Amaranthaceae,Solanacae, Leguminosae and Ranunculaceae. *Chenopodium quinoa* and *C. amaranticolor* showed chlorotic local lesions. *Gomphrena globosa* developed necrotic local lesions. Systemic symptoms were produced in the members of Cucurbitaceae and *Ranunculus sardous.* Z2, Z4 and Z5 caused mosaic symptoms on *Phaseolus vulgaris* cv. Red Kidney and *P. vulgaris* cv. Khorram but Z1 and Z3 caused chlorotic local lesions.Virus was purified from *Cucurbita pepo.* Virus particles in immunoelectron microscopy were filamentous flexuous. The molecular weights of coat protein using SDS-PAGE and western blotting were estimated at 32 kDa. Reverse transcription polymerase chain reaction (RT-PCR) was performed using one primer pairs designed by Desbiez *et al.* An approximately 458 bp fragment was amplified with a specific primer.**

Keywords: ELISA, RT-PCR, SDS-PAGE, ZYMV.

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV), a potyvirus with a worldwide distribution, was first discovered in Italy (Lisa *et al.*, 1981). In Iran, ZYMV is known to occur in squash fields in Tehran Province (Ghorbani, 1988).

This virus can cause severe economic losses in many cucurbits. ZYMV belongs to the genus potyviruses in the Potyviridae, a group of plant viruses characterized by a monoparticle, positive-sense, single stranded RNA genome encapsidated in flexuous rod shape particles. Viral RNA consists of about 9600 nucleotides (Balint *et al*., 1990) with a 5′ viral protein genome linked (VPG) and a poly (A) tail (Dougherty *et al.*, 1993). Like other potyviruses, ZYMV is efficiently transmitted by aphids in a nonpersistent manner (Lisa *et al.*, 1981). Control of ZYMV is difficult but the use of resistant cultivars, inoculation of mild ZYMV-WK strains for cross protection against severe challenging strains (Walkey *et al.*, 1992; Lecoq *et al.*, 1991) and the use of mineral oil sprays (Makkouk *et al.*, 1986) in association with pyrethroids (Raccah *et al.*, 1985), might provide protection under certain ecological conditions.

MATERIALS AND METHODS

Sample Collection

Samples were collected during the 2002 and 2003 growing seasons from field-grown squash (*Cucurbita pepo*) in Tehran Province. In this region, squashes are planted during early April and harvested from June

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to July. Virus infections become visible after the setting of the first fruits. Infected plants show symptoms such as: mosaic, yellowing, leaf distortion, shoestring, fruit deformation and yield reduction. Young leaves from some symptomatic plants were collected at random. All samples were kept in ice chests for transportation to the laboratory. Each plant sample was kept separately in a plastic bag at 4°C until analyzed.

Virus Identification

DAS-ELISA (double antibody sandwich ELISA) as described by Clark and Adams (1977) was used with a polyclonal antiserum against ZYMV (DSMZ-AS0239). A 200 µl aliquot of IgG was added to coat each well of plates. Each step of ELISA was followed by a 4-hr incubation at 37°C or a 12-hr incubation at 4°C. This was followed by three washes with a washing buffer. Ten milliliters of sample buffer, pH 7.4, was added to 1g tissue samples that had been ground in liquid nitrogen, and 200 µl of this extracted was added to each well. The reaction was read using a colorimeter at 405 nm after adding conjugate incubation with substrate for about one hour.

Host Range Studies

Five original isolates (Z1, Z2, Z3, Z4 and Z5) recovered from infected squash plants in the host range studied (from different locations) were maintained in *Cucurbita pepo* by sap inoculation. For plant assays, 27 species from 6 families were inoculated with the virus isolates. Sap prepared from leaves which were in 0.01 M Sodium phosphate buffer, pH 7, was rubbed onto leaves dusted with carborundum powder. The Leaves were then rinsed with water, and plants were maintained in an insect–proof screen house for observation, Symptoms on both inoculated and upper, uninoculated leaves were recorded. Tests for latent infection were conducted by back–inoculation to either *Chenopodium amaranticolor* Cost and Reyn or *C. quinoa* Wild.

Virus Purification

The virus was purified according a methods described by Purcifull and Hiebert (1979) and was purified from systematically infected tissue of *Cucurbita pepo*. All stages of the purification were completed at 4°C. Tissue was homogenized (1:2w/v 400g/ 800ml) in a 0.5 M potassium phosphate buffer, pH 7.2, containing 2 g Na_2SO_3 and a 1:1(v/v) mixture of chloroform and carbon tetrachloride.

The filtrate of the homogenate after passing it through eight layers of cheese cloth, was given low–speed centrifugation at 4000 g for 5 minutes. The aqueous phase was collected and centrifuged at 12000 g for 20 minutes to the resultant supernatant. Polyethylene glycol (PEG) (mol wt 6000) to concentration was added to 8%, and the preparation was stirred for one hour. The mixture was centrifuged at 12000 g for 10 minutes. The precipitate was resuspended in small volume of buffer. Virus purity was determined by UV spectroscopy. A_{260}/A_{280} ratio was determined.

Protein Analysis

The molecular weight of the viral coat protein was estimated by SDS PAGE (Sodium dodecyl sulfate poly acryl amid gel electrophoresis) as described by Lamemmli (1970).

A purified virion suspension and plant samples infected with the virus were mixed with the sample buffer, boiled at 100 °C for 5 minutes and subjected to electrophoresis on 12% poly acryl amid gel along with poly peptide size standard. The gel was stained with coomassie blue. The molecular weight of polypeptides from ZYMV was determined by comparison with the size standards.

For western blot analysis, the purified virus and infected plant samples were electro-

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phoresed as described previously. The separated proteins were transferred to nitrocellulose using the buffer styles of Sambrook *et al*. (1989). Probing of the western blots was done as described by Burgermeister and Koeing (1984) using the ZYMV antiserum to ZYMV (DSMZ DAS-0234) at a dilution of 1:1000 (v/v).

Electron Microscopy

Immunoelectron microscopical decoration tests was carried out at DSMZ (German collection of microorganism and cell cultures plant collection) in Germany.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from ZYM-infected plants was extracted using a phenol/chloroform protocol (Wadsworth *et al.*, 1988). Three µl of RNA were submitted to reverse transcription in a final volume of 20 µl, using 2µl PCR buffer 10x (0.5 M Tris-HCl, 0.7 M KCl, 0.1 M $MgCl_2$, pH 8), 1µl DTT (100 mmol/µl), 1µl dNTPs (10 mmol/ µl), 0.5 µl RNase inhibitors enzymes (10 mmol/µl) and 2 µl Reverse-DAG primer (100 pmol /µl) (5 /- GCG TGG CAA TGA CAT - 3/: nucleotide position 8735-8749 on sequence L 31350) for one hour at 42°C with 0.5 µl MMLV reverse transcriptase (200 mmol/µl). 5 µl of the RT reactions were used for PCR using a 5 µl PCR buffer 10x, 2 µl $MgCl_2$, 1 µl dNTPs (10mmol/µl), 0.5 µl Taq polymerase (5 unit/µl), 1 µl Reverse-DAG (100 pmol) and 1 µl Forward-DAG (100 pmol) (5 /- ATT TGC GCT GCG ATG-3/: 8291-8305 on sequence L 31350) oligonucleotides encompassing the N-terminal part of the coat protein coding region and the C-terminal part of the polymerase (NIb) (primers designed by Desbiez *et al.*, 2002). PCR reactions were performed by a first denaturation of the samples at 94°C for 3 minutes followed by 35 cycles at 94°C for 30 seconds, 43 °C for 30 seconds and 72°C for 30 seconds and a final elongation step at 72°C for 7 minutes. PCR products were controlled by electrophoresis on 1% agarose gel (Desbiez *et al*., 2002).

RESULTS

Host Range

The virus was isolated from infected squash species from the families Cucurbitaceae, Amaranthaceae, Chenopodiaceae and Legumonosae but it did not infect the numbers of Solanaceae that were tested. Mosaic, blistering, leaf distortion and stunted growth were observed on *Cucurbita pepo* cv. Zucchini (figures 3 and 4), *C. Pepo* cv. Maragheh, cv. Khoy, *Cucumis melo* cv. Asgrown. Necrotic local lesions were found on *Gompherena globosa* and chlorotic local lesions with symptoms of systemic infection on *Chenopodium quinoa*, *C. amaranticolor* and *Phaseolus vulgaris* cv. Khorram and cv. Red kidney were all observed (Figures 1 and 2). Latent infection was detected in *Ranunculus sardous* (Table 1).

Fgiure 1. Symptoms of mosaic on *Phaseolus vulgaris* cv. Red Kidney.

Figure 2. Symptoms of chlorotic local lesions on *Phaseolus vulgaris* cv. Red Kidney.

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Figure 3. Symptoms of leaf deformation on *Cucurbita.pepo.*

Figure 4. Symptoms of blistering on *Cucurbita pepo*

Virus Purification and Characterization

Greenhouse plants with the highest absorbance values determined in ELISA were chosen to purify the putative ZYMV. Appromixately 16.2 mg of purified ZYMV was obtained from every 1 g of 8 day-old infected pumpkin leaves. Purification of the virus using the protocol described by Purcifull and Hiebert (1979) was successful. Its A_{260}/A_{280} ratios were 1.28.

ISEM

Electron microscopy analysis showed flexuous filamentous particles typical of the Potyviridae morphology (Figure 5).

Table 1. Reaction of selected indicator plant species to ZYMV.

Families	Test plants	Symptoms in leaves
Cucurbitaceae	*Cucurbita pepo* cv. Zucchini	m, b, Id
	C. pepo cv. Khoy	m, b, Id, ss
	C. pepo cv. Maragheh	m, b, Id, ss
	Cucumis melo cv. Asgrown	b, m
	C. melo cv. Melon Seed	b
	C.melo Local cultivar	b
	C. sativus cv. Dominus	m
	C.sativus cv. PS	m
	Citrullus lanatus cv. Crimson Sweet	s, m, b
	Luffa acutangula	m
	Gomphrena globosa	nll
Amaranthaceae	*Amaranthus paniculatus*	-
	Chenopodium quinoa	cll
Chenopodiaceae	*C. amaranticolor*	cll
	Spinacia oleraceae cv. Keshtzar	-
	Phaseolus vulgaris cv. Khorram	cll , m
	P.vulgaris cv. Daneshkadeh	-
Leguminoseae	*P.vulgaris* cv. Red Kidney	cll , m
	P. vulgaris cv. Bountiful	-
	Vigna unguiculata	-
	Vicia faba	-
	Pisum sativum	-
Solanaceae	*Dautura metel*	-
	D. stramonium	-
	Nicotiana tabacum	-
	N. glutinosa	-
	N. benthamiana	-
Ranunculaceae	*Ranunculus sardous*	-

Ss= Shoe string, id= Leaf distortion, b = Blistering, s= Stunted growth, m= Mosaic
1= Latent infection, cll= Chlorotic local lesion, nll= Necrotic local lesion.

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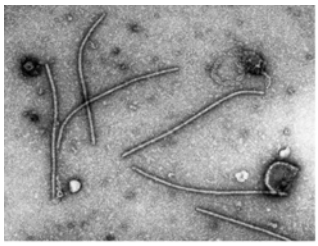

Figure 5. Immunosorbent electron microscopy of virus (8000X) (Stained with uranyl acetate).

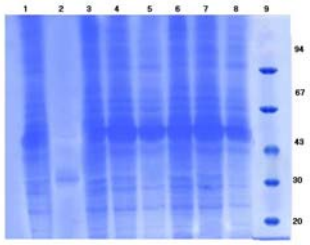

Figure 6. Denaturing polyacrylamide gel electrophoresis of infected plants (line 1, 4, 6), purified virus (line 2), healthy plant (8, 5, 3, 7) and molecular weight marker proteins (line9).

SDS-PAGE and Western Blot

Molecular weight of the capsid protein was determined as 32 kDa. Western blot analysis also revealed one band of approximately 32 kDa in the purified virus preparation and infected plant samples, while no band was found in the healthy plant extracts (Figures 6 and 7).

RT-PCR

RT-PCR was carried out using the primers DAG/F and DAG/R (described previously) which resulted in a fragment of 458 nts (Figure 8).

DISCUSSION

Squash (*Cucurbita pepo)* belongs to the Cucurbitaceae family and is one of the most important economic crops in the world. Cucurbitaceae are threatened by viruses and there are many viruses that damage this family. ZYMV is a *potyvirus* with a worldwide distribution that causes serious economic losses in many cucurbits.

ZYMV was first found in squash in Tehran (Ghorbani, 1988) and then was discovered in 2000 in Mazandaran (Delkhosh *et al.*).

The results of the host range studies, ELISA, electron microscopy and RT-PCR indicate that ZYMV isolates from Iran share the reported properties of the ZYMV. In direct caparison with ZYMV, very similar reactions were recorded amongst the isolates of ZYMV from Iran. The isolates induced systemic shoestring symptoms without local

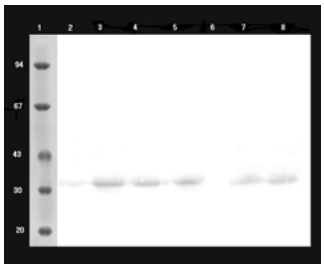

Figure 7. Electro blot immunoassay (Western blotting) of capsid protein of ZYMV with antiserum to ZYMV. Figurs on left indicate the position and the molecular weights of the markers protein, lane 1: marker proteins, 2, 4, 7, 8 infected plants 3, 5 purified virus and 6 healthy plant (Marker related previous step).

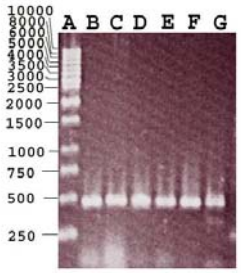

Figure 8. 1% agarose gel electrophoresis analysis of RT-PCR products amplified with DAGF/DAGR primer pair .Lanes B-G lane A ladder.
(B= Z1, C= Z2, D= Z3, E= Z4, F=Z5, G= positive control).

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lesions.

However, some differences in the host range of Iranian isolates were observed in which Z1 and Z3 isolates induced local chlorotic lesions on *Phaseolus vulgaris* cv. Khorram and cv. Red Kidney. This is rare and only ZYMV-Conecticut, - France, - Taiwan induced these kinds of symptoms. Z2, Z4 and Z5 isolates induced mosaic in *Phaseolus vulgaris* cv. Khorram and cv. Red Kidney and these symptoms are different from other known strains.

These isolates could systemically infect Cucurbitaceous plants without induced local lesions. ZYMV isolates induced shoestring symptoms in *Cucurbata pepo*. This symptom has also been described by Wong (1994) in Singapore.

Nicotiana benthamiana, N. tabacum, N. glutinosa and Pisum sativam were immune to ZYMV isolates. *Ranunculus sardous* induced latent infection and was used to as differential host (Wong, 1994).

Our SDS-PAGE pattern showed the presence of a protein of molecular mass 32 kDa, the expected size for the CP from ZYMV previously reported (Wong *et al.*, 1994).

This 32 kDa protein was recognized by anti-ZYMV antibodies in Western blot assays, confirming the identity of the isolated viral particle as a potyvirus immunogenically related to ZYMV.

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تعیین خصوصیات ویروس موزائیک زرد کدودر مزارع کدو مسمایی استان تهران

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چکیده

ویروس *Zucchini yellow mosaic virus* (ZYMV) یکی از ویروسهای مهم مخرب کدوئیان می‌باشد. طی دو سال ۸۲-۱۳۸۱، از مزارع کدو مسمایی استان تهران تعدادی نمونه جمع‌آوری شد. گیاهان آلوده نمونه برداری شده دارای علائم مختلف از جمله: موزائیک، زردی، بدشکلی شدید برگ و بدشکلی میوه بودند. برای شناسایی ویروس *Zucchini yellow mosaic virus* (ZYMV) از آزمون DAS-ELISA استفاده شد. به منظور تعیین دامنه میزبانی ویروس، ۲۷ گونه گیاهی از ۶ خانواده (Cucurbitaceae ، Amaranthaceae ، Solanaceae ، Ranunculaceae ، Leguminosae ، Chenopodiaceae) به صورت مکانیکی با ۵ ایزوله (Z1 ،Z2 ،Z3 ،Z4 و Z5) مایه‌زنی شد. تمام ایزوله ها روی سلمه‌تره (*Chenopodium quinoa* , *C. amaranticolor*) لکه‌های موضعی کلروتیک وروی گل تکمه‌ای (*Gomphrena globosa*) لکه‌های موضعی نکروتیک ایجاد کردند و روی گیاهان خانواده کدوئیان و *Ranunculus sardous* سیستمیک شدند. ایزوله های Z2 ،Z4 و Z5 بر روی لوبیا چیتی رقم خرم و لوبیا رقم Red Kidney علائم موزائیک ایجاد کردند ولی دو ایزوله دیگر روی این دو رقم لوبیا بدون آنکه سیستمیک شوند، لکه‌های موضعی کلروتیک به وجود آوردند. ایزوله Z1 ویروس روی کدو مسمایی تکثیر و خالص‌سازی شد. پیکره های ویروس در روش ایمنوالکترون میکروسکپی به صورت رشته های انعطاف پذیر مشاهده شد. وزن پروتئین پوششی با آزمون SDS - PAGE و وسترن بلات ۳۲ کیلودالتون تعیین گردید. روش RT- PCR با استفاده از یک جفت پرایمر که توسط Desbiez و همکاران (۲۰۰۲) طراحی شده بود، انجام شد و قطعه ای به طول ۴۵۸ جفت بازتکثیر شد.

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