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IDENTIFICATION OF VIRUSES INFECTING PUMPKINS (*Cucurbita pepo* L.) IN SERBIA

ABSTRACT: This study was carried out in order to identify the major viruses infecting pumpkins (*Cucurbita pepo*) grown in Serbia. Leaf samples from virus-infected pumpkin plants were collected in mid-July 2001. Naked-seeded and hulled oil pumpkins, patty pan, zucchini and summer squash from three different locations were included (Table 1). Virus-infected plants showed different symptoms (Table 2 and Figures 1—4). Due to the great variability of the symptoms, the causal viruses could not be fully and precisely determined by visual examination only.

The infected samples were tested by the biotest, as well as by two serological methods, ELISA and EBIA. Polyclonal antibodies raised against cucumber mosaic cucumovirus (CMV), zucchini yellow mosaic potyvirus (ZYMV), watermelon mosaic potyvirus 1 (WMV-1), watermelon mosaic potyvirus 2 (WMV-2) and squash mosaic comovirus (SqMV) were used. In each of the 50 collected samples one or two viruses were detected (Tables 3 and 4). The most prevalent viruses infecting pumpkins were ZYMV (62%) and CMV (58%). WMV-2 was extremely rare.

KEY WORDS: *Cucurbita pepo*, pumpkins, plant viruses, zucchini yellow mosaic potyvirus, cucumber mosaic cucumovirus, watermelon mosaic potyvirus 1, watermelon mosaic potyvirus 2, biotest, ELISA, EBIA

INTRODUCTION

Viruses are the most important pathogenes of cucurbits (cucumber, watermelon, melon and pumpkins) belonging to the *Cucurbitaceae* family. More than 30 infectious viruses causing destructive symptoms and considerable economic losses were reported on these plants (Zitter et al., 1996). Their occurrence, spreading, intensity of infection and destructiveness depend on complex interrelations between the virus, its host plant, the vectors and the environment. It is usually not easy to find appropriate control measures to reduce the extent of destruction.

In order to reduce the harmful effect of a viral disease under field conditions, it is necessary to select and apply appropriate control measures. The first step in this direction is collecting infected plant parts from different locations and from various host genotypes, followed by the development of reliable methods of diagnosing.

Very few researches on cucurbit viruses have been carried out in Yugoslavia (Stakić and Nikolić, 1966; Pejčinovski, 1978; Tošić et al., 1996). Recently, a serious virus infection of pumpkin (*Cucurbita pepo* L.) has been reported by Dukić et al. (2001) for the location of Veliko Selo. The virus was identified as zucchini yellow mosaic potyvirus (ZYMV), known to be one of the most destructive viruses of pumpkins. Further investigation on virus diseases of pumpkins in Serbia thus became necessary. This paper, describing some of the results of our project aimed at studying the virus diseases of pumpkins in Serbia, with special reference to oil pumpkins, is a part of it.

MATERIAL AND METHODS

Collection of infected plant material

Samples of virus-infected pumpkin plants were collected in mid-July 2001 from naked-seeded and hulled oil pumpkins, patty pan, zucchini and summer squash, at three different locations (Table 1). The collected plant material consisted of young leaves and fruits from individual plants showing distinct symptoms of virus infection on the leaves, as well as at the level of the overall appearance of the plant. Each sample represented a single plant. The plants and their corresponding leaf samples were numbered 1—50 for later identification.

Tab. 1 — Samples collected from the virus-infected plants of pumpkin (*C. pepo*) in the field in 2001

Locality	Pumpkin type and cultivar (variety)	Designation of sample
Bački Petrovac	Breeding material of naked-seeded and hulled oil pumpkin	1—8
	Naked-seeded oil pumpkin cv. „Olinka”	9—14
Srbobran	Hulled oil pumpkin cv. „Olivija”	15—16
Torda		42—50
	Naked-seeded oil pumpkin cv. „Olinka”	17—24
	Patty pan cv. „Eva”	25—29
	Zucchini cv. „Zita”	30—36
	Summer squash cv. „Beogradska”	37—41

Pictures of the sampled plants were taken at the time of collection and the symptoms were described in written.

The collected samples were stored at 4°C, until the investigation on the viral nature of the symptoms and the identification of the viruses by the biotest and serological analyses were done.

Biotest

The infectious nature of the disease and the biological characterization of the isolated viruses were performed by mechanical inoculation. Young leaves expressing virus symptoms and surface tissues of the warted fruits were homogenized in cold 0.01 M phosphate buffer of pH 7.0 at the ratio 1:1. The following test plants were used for virus isolation and determination based on the provoked symptoms: *Chenopodium amaranticolor*, *C. quinoa*, *C. foetidum*, *Vigna sinensis*, *Citrullus lanatus* cv. „Crimson sweet", *Cucumis melo* cv. „Ananas", *Cucumis sativus* cv. „Pariski kornišon", *Cucurbita pepo* cv. „Beogradska", *Luffa* sp., *Lagenaria* sp., *Nicotiana tabacum* var. Samsun, *N. glutinosa*, *N. clevelandii* and *N. benthamiana*. The leaves of the test plants were covered with carborundum powder of 400 mesh, followed by rubbing the plant sap into the leaves of the test plants. Two plants of each test plant species were used for mechanical inoculation. The inoculated test plants were kept in glasshouse conditions and checked for symptom development at two-day intervals, up to one month after inoculation.

Serological analyses

All the collected plant samples were tested for virus identification by EBIA (Western blot) according to the method described by O'Donell et al. (1982) and modified by Hewish et al. (1986). The antigens required for EBIA were prepared from the extract of the collected leaves by the method of Laemmli (1970). Polyclonal antibodies (produced by Bioreba AG, Switzerland) raised against cucumber mosaic cucomovirus (CMV), Zucchini yellow mosaic potyvirus (ZYMV), Watermelon mosaic potyvirus 1 (WMV-1), Watermelon mosaic potyvirus 2 (WMV-2) and Squash mosaic comovirus (SqMV) were used at 1:1000 dilution. Goat antirabbit antibodies (produced by Bio-Rad Lab., Richmond, CA, USA) were diluted 1:2500 in skimmed milk. The occurrence of blue-pink color on nitrocellulose membrane was considered as the sign of positive, and its absence as a negative reaction. The molecular weight of the protein subunit of the virus was determined by Prestained SDS-PAGE Standards-Low Range (produced by Bio-Rad Lab., Richmond, CA, USA).

In addition, the samples designated by numbers 2, 6, 10, 14, 16, 20, 27, 31, 32, 36, 38, 41, 45, 46, 48 i 49 were also tested serologically by ELISA test, using polyclonal antisera produced against the following viruses: CMV, ZYMV, WMV-1, WMV-2 and SqMV. For the serological evidence of the viruses the standard direct ELISA (DAS-ELISA), based on the procedure of Clark and Adams (1977), was used with commercial kits of specific antibodies and alkaline phosphatase-labelled conjugate γ-globuline (produced by

Bioreba AG, Switzerland) at 1:1000 dilution in corresponding buffer. Plant extracts for ELISA analysis were ground in the extraction buffer at 1:4 ratio. The reaction was considered positive if the absorption of light at 405 nm was at least twice as high compared with the absorption of the corresponding control.

RESULTS

Symptoms on infected plants under field conditions

Visual inspection of the infected plants revealed various symptoms ranging from mild mosaic, yellowing, spotting and mottling to deformation of leaf lamina. The observed symptoms were classified into 11 symptom categories (Table 2). In many cases simultaneous occurrence of different symptoms was observed on the same plant. Some plants showed virus symptoms only on some of their stems, or on young leaves only. The most frequent symptoms were the deformation of leaf lamina, yellow-green mosaic of different intensity and blistering of leaf lamina.

Tab. 2 — Categories of symptoms on infected plants in the field

Symptoms category	Description of the symptoms
1	mild mosaic
2	yellow-green mosaic
3	yellowing of leaves
4	chlorotic spotting
5	chlorotic mottling
6	netlike mosaic
7	green veinbanding
8	blistering of leaf lamina
9	deformation of leaf lamina
10	plant stunting
11	knobbed fruits

Results of biotest

Based on the reaction of test plant species provoked by the isolated viruses, it could be concluded that the tested plant material was infected by ZYMV, CMV and WMV-2 (Table 3).

Tab. 3 — Reaction of test plants to mechanical inoculation with ZYMV, CMV and WMV-2

Test plant species	Symptoms*					
	ZYMV		CMV		WMV-2	
	local	systemic	local	systemic	local	systemic
Chenopodium amaranticolor	LLc	—	LLn	—	LLn	—
Chenopodium quinoa	LLc	—	LLn	—	LLc	M, D
Chenopodium foetidum	—	—	LLn	M	—	—
Vigna sinensis	—	—	LLc	—	—	—
Citrullus lanatus cv. Crimsonsweet	—	M	LLc	M	—	M
Cucumis melo cv. Ananas	—	M, D	—	M, D	—	M, D
Cucumis sativus cv. Pariski kornišon	—	M	—	M	—	M
Cucurbita pepo cv. Beogradska	—	M, D	—	M, D	r, LLc	M, D
Luffa sp.	—	—	—	M	—	—
Lagenaria sp.	—	M	—	M	—	M
Nicotiana tabacum var. Samsun	—	—	—	M	—	—
Nicotiana glutinosa	—	—	—	M	—	—
Nicotiana benthamiana	—	—	—	—	—	—
Nicotiana clevelandii	—	—	—	M	—	—

* —: no symptoms
LLc: chlorotic local lesions
LLn: necrotic local lesions
M: mosaic
D: deformation of leaves
r: infrequent appearance of lesions

All isolates of each of the investigated viruses had the same host range and caused the same type of symptoms on them. Local symptoms typically appeared about 5—7 days after inoculation, except for local chlorotic spots caused by the isolates of ZYMV on *C. quinoa* and *C. amaranticolor*, which appeared considerably later, 10 days after inoculation.

Some of the test plants exhibited rather characteristic types of reaction that could be useful for identification and differentiation of mechanically transmissible viruses of pumpkins. CMV caused systemic mosaic symptoms on the plants of the genus *Nicotiana* sp., except for *N. benthamiana*, as opposed to ZYMV and WMV-2, which were not infectious for this genus. Of the three viruses identified, only CMV was infectious for *Vigna sinensis*, causing local chlorotic spots, as well as for *C. foetidum* on which local necrotic spots could be observed along with systemic infection. On *C. quinoa*, CMV caused local spots that changed to necrosis rapidly, in a few days. Contrary to the other two viruses, CMV induced systemic infection on *Luffa* sp. expressed as mosaic with ringlike patterns. Of the three viruses studied, only CMV caused mosaic combined with large local chlorotic spots on watermelon. ZYMV and WMV-2 showed the identical host range, but could be differentiated from each other by the reaction on *C. quinoa* and *C. amaranticolor*. On *C. quinoa*, WMV-2 caused local chlorotic spots and easily distinguishable mosaic combi-

ned with slight deformation of the leaf lamina. At the same time WMV-2 caused local chlorotic spots on *C. amaranticolor* which turned to necrosis after a few days, contrary to ZYMV which provoked chlorotic spots that remained chlorotic till the full collapse of the leaf.

The presence of infection caused by a single virus or by different combinations of the three viruses was indicated by the occurrence of characteristic symptoms on the test plants. Infection caused by a single virus was registered in 34 samples (68% of the total sample number), a combined infection by two viruses was determined in 16 samples (32%). ZYMV was determined in 31 samples (62%), out of which 16 samples (32%) were single infections, 14 samples (28%) showed a mixed infection by ZYMV and CMV, and one sample (2%) showed a mixed infection by ZYMV and WMV-2. CMV was detected in 29 samples (58%), out of which 14 samples (28%) showed single, and 15 samples (30%) mixed infection. As far as the combined infections of CMV were concerned, CMV was found in combination with WMV-2 in only 1 sample (2%). WMV-2 was detected in only 6 samples (12%), 4 samples (8%) being single infections. None of the samples were simultaneously infected by all three viruses.

Results of serological analysis

The results of the serological tests were in accordance with the biotest results. Table 4 presents the combined results of the biotest, EBIA and ELISA.

Using the EBIA method and polyclonal antibodies, it was possible to confirm the presence of ZYMV and WMV-2 in the same samples in which they were detected by the biotest. By comparing with the markers of known molecular weight, the molecular weight of protein subunits of these two viruses was estimated at 35 000, which is in accordance with the results of Purcifull et al. (1984). It could be observed in Table 4 that antibodies specific for CMV caused a positive reaction only in samples 18, 30, 33, 34, 41 and 42, in which this virus was isolated from test plants also detected by using the biotest. It was demonstrated by very pale-colored strips on nitrocellulose paper.

All 16 samples tested by the ELISA showed specific reaction with homologous, and absence of positive reaction with heterologous antisera. The presence of viruses in the samples was therefore reliably confirmed, regardless of the infection being single or mixed.

When polyclonal antibodies specific to WMV-1 and SqMV were applied, no positive serological reactions were observed either by the EBIA or the ELISA test.

Relationships between the isolated and identified viruses on one side and the symptom types exhibited in the field on the other are shown in Table 4. ZYMV was isolated from samples with all symptom types, but CMV showed only 9 out of the 11. From infected plant parts showing symptom type 1, 5 and 8, ZYMV or CMV or WMV-2 were isolated alone, but in some cases also complexes of CMV with WMV-2 were determined. Complexes of ZYMV and CMV resulted in symptom types 8 or 5.

Tab. 4 — Viruses identified and their symptom categories on infected plants in the field

Sample	Virus identified*	Symptoms category**
1	CMV[1]	4
2	CMV[1, 3]	2, 9
3	CMV[1]	1, 9
4	CMV[1]	1, 8
5	CMV[1]	2, 8
6	CMV[1, 3]	3, 4, 7, 8
7	WMV-2[1, 2]	1
8	CMV[1]	3, 8
9	WMV-2[1, 2]	1, 8
10	CMV[1, 3], WMV-2[1, 2, 3]	1, 8
11	WMV-2[1, 2]	1
12	CMV[1]	2
13	CMV[1]	1
14	CMV[1, 3]	2, 8, 9
15	ZYMV[1, 2]	4, 8
16	CMV[1, 3]	4, 8
17	ZYMV[1, 2]	1, 2, 3
18	CMV[1, 2], ZYMV[1, 2]	2, 8
19	ZYMV[1, 2]	3, 7
20	ZYMV[1, 2, 3]	3, 9
21	WMV-2[1, 2]	5
22	ZYMV[1, 2]	11
23	ZYMV[1, 2]	1, 9
24	ZYMV[1, 2]	1, 9
25	ZYMV[1, 2]	1, 7
26	ZYMV[1, 2]	1, 3, 7
27	CMV[1, 3], ZYMV[1, 2, 3]	2, 8, 9, 11
28	ZYMV[1, 2]	2, 5, 10
29	CMV[1]	9, 10
30	CMV[1, 2], ZYMV[1, 2]	2, 9
31	WMV-2[1, 2, 3] ZYMV[1, 2, 3]	2, 9
32	ZYMV[1, 2, 3]	2, 9
33	CMV[1, 2], ZYMV[1, 2]	3, 9
34	CMV[1, 2]	5, 9
35	CMV[1], ZYMV[1, 2]	5, 9
36	CMV[1, 3], ZYMV[1, 2, 3]	9, 10
37	CMV[1]	5, 7
38	CMV[1, 3], ZYMV[1, 2, 3]	3, 7
39	ZYMV[1, 2]	5, 7
40	ZYMV[1, 2]	6, 7
41	CMV[1, 2, 3], ZYMV[1, 2, 3]	5, 6, 7

Sample	Virus identified*	Symptoms category**
42	CMV[1, 2], ZYMV[1, 2]	3, 5
43	CMV[1], ZYMV[1, 2]	3, 6, 7
44	CMV[1], ZYMV[1, 2]	3, 7
45	CMV[1, 3], ZYMV[1, 2, 3]	2, 8
46	CMV[1, 3], ZYMV[1, 2, 3]	2, 8, 9
47	CMV[1], ZYMV[1, 2]	3, 7
48	ZYMV[1, 2, 3]	2, 8, 9
49	ZYMV[1, 2, 3]	1, 3
50	ZYMV[1, 2]	2, 8, 9

* Virus identification by 1: biotest, 2: EBIA, 3: ELISA
** Designation for symptom categories from Table 2.

DISCUSSION

Members of the *Cucurbitaceae* family are highly sensitive to virus infection. They are infected by more than 30 viruses, the most important being: Cucumber mosaic cucumovirus (CMV), Watermelon mosaic potyvirus 2 (WMV-2), Zucchini yellow mosaic potyvirus (ZYMV), Watermelon mosaic potyvirus 1 (WMV-1, earlier: Papaya ringspot virus, PRSV) and Squash mosaic comovirus (SqMV) (Zitter et al., 1996).

The investigation reported in this paper confirms the presence of ZYMV, CMV and WMV-2 in our country. These viruses had been described previously in other locations (Dukić et al., 2001). ZYMV and CMV could be considered as widespread. The most frequent virus, ZYMV, was present in 62% of samples. This virus occurred in a large number of samples (30%) in combination with CMV. Compared with the other two viruses, WMV-2 was detected only sporadically.

The symptoms caused by these three viruses in different pumpkin types and cultivars were various. It was not possible to establish a correlation between the type of symptom and the virus, which was an indication that field symptoms cannot be used as reliable indicators, even in the case when infection is caused by a single virus.

Having on mind that the investigations of cucurbit viruses in Serbia have started recently (Dukić et al., 2001), it was necessary in this investigation to study not only the occurrence of viruses in different locations but also some biological characteristics of the isolated viruses. The gathered results should facilitate further diagnosing and monitoring of the viruses of the cucurbits.

Based on the species of host plants and characteristic symptoms it is possible to make a biological characterization of mechanically transmissible viruses of cucurbits. CMV is easiest to prove and differentiate from other viruses, based on its specific reactions on the plants of *Nicotiana* spp. It is also relatively easy to detect WMV-2 in the presence of ZYMV, based on the systemic reaction on *C. quinoa*. ZYMV is not hard to detect in case of single infection, but in the case of mixed infection with WMV-2, detection is possible only ba-

sed on local chlorotic sposts on *C. amaranticolor*. In order to make the detection of ZYMV in mixed infections possible, it is necessary to find a host plant displaying a specific reaction only to ZYMV.

The results of the biotest showed no observable differences among the isolates of the same virus, indicating the lack of variability within individual viruses under the conditions maintained in this study. The isolates of ZYMV as well as of WMV-2 showed very similar but not identical reactions to those published in the literature.

None of our isolates of ZYMV were infectious for *Luffa* sp., although numerous literature data dealing with ZYMV characterization referred to isolates capable of infecting *Luffa acutangula* (Lisa et al., 1981; Lisa and Lecoq, 1984; Provvidenti and Gonsalves, 1984; Prieto et al., 2001) and *Luffa aegyptica* (Lisa et al., 1981). The ZYMV isolate from cucumber described as not infectious to *Luffa acutangula*, differed from our isolates by reaction to *N. benthamiana* (Lesemann et al., 1983).

The isolates of WMV-2 obtained in this study tended to cause the same symptoms as those previously described in literature (Provvidenti and Schroeder, 1970; Purcifull et al., 1984). However, they were not infectious to *N. benthamiana*, which was not in accordance with the results of other authors (Purcifull and Hiebert, 1979; Tobias and Tulipan, 2002), and which made them different from WMV-2 derived from cucumber by Tošić et al. (1996). At the same time, our isolates of WMV-2 caused numerous chlorotic spots on the infected leaves of *C. quinoa*, just as described for most isolates of the same virus, but, contrary to others, our isolates belonged to a small group of isolates capable of systemic infection of this test plant and of causing mosaic and leaf deformation (Lisa and Dellavalle, 1981, Purcifull et al., 1984, Tošić et al., 1996).

The symptoms on test plants induced by our CMV isolates were not much different from those described by other authors (Lastra, 1968; Cohen and Nitzanny, 1963; Tobias and Tulipan, 2002).

The identification of the viruses collected in this study was confirmed by serological methods using appropriate antisera. The ELISA method, used worldwide for routine detection of cucurbits viruses (Menassa et al., 1986; Yuki et al., 2000), appeared to be very sensitive and appropriate for the study of a large number of samples. The EBIA method showed to be suitable for the detection of ZYMV and WMV-2, but for the detection of CMV it is necessary to standardize and increase the sensitivity of this method, in order to make it suitable for the cases when the virus occurs in low concentrations.

Based on our result it could be concluded that serological testing of a large number of samples, especially by the ELISA test, is sufficiently sensitive and appropriate for the detection of the presence of ZYMV, CMV and WMV-2 in cucurbits.

In spite of the fact that viruses cause numerous and very destructive diseases on the cultivated species from the family *Cucurbitaceae*, little attention has been paid to these viruses in our country in the past. In view of the intensified incidence of cucurbit viruses and their growing economic importance in Serbia, it is necessary to continue this study, focusing the attention on ZYMV, one of the most destructive viruses of cucurbits.

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ИДЕНТИФИКАЦИЈА ВИРУСА ИНФЕКТИВНИХ ЗА ОБИЧНУ ТИКВУ (*Cucurbita pepo* L.) У СРБИЈИ

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Резиме

Циљ ових истраживања био је да се идентификују најважнији вируси тикава (*Cucurbita pepo* L.) гајених у Србији. Узорци биљног материјала уљане тикве-голице, уљане тикве са љуском, тиквице за јело, патисона и цукинија, који су били заражени вирусима, сакупљени су у три локалитета средином јула 2001. године (таб. 1). Биљке заражене вирусима показивале су различите симптоме (таб. 2 и сл. 1—4). Тачна детерминација вируса само на основу симптома није могућа због варијабилности самих симптома.

Заражени узорци су тестирани биотестом као и применом две серолошке методе, ELISA и EBIA коришћењем поликлоналних антитела на Cucumber mosaic cucomovirus (CMV), Zucchini yellow mosaic potyvirus (ZYMV), Watermelon mosaic potyvirus 2 (WMV-2), Watermelon mosaic potyvirus 1 (WMV-1) и Squash mosaic comovirus (SqMV).

У 50 испитаних узорака детектован је један или два вируса (таб. 3 и 4). Преовлађујући вируси тикава били су ZYMV (62%) и CMV (58%). WMV-2 је детектован у веома малом броју узорака.

Fig. 1. — Intensive yellow-green mosaic and chlorotic mottling of leaf (sample 28) caused by zucchini yellow mosaic potyvirus

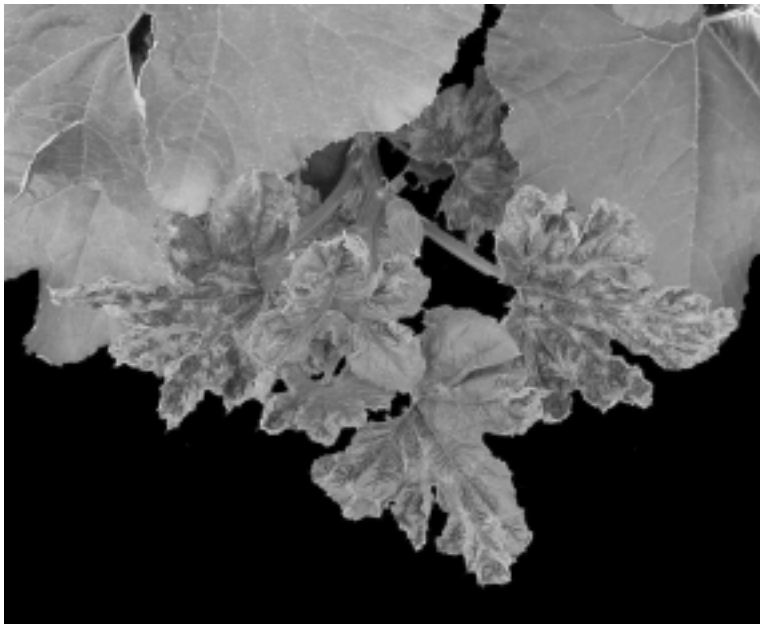

Fig. 2 — Yellow-green mosaic and blistering of leaf lamina (sample 2) caused by cucumber mosaic cucumovirus

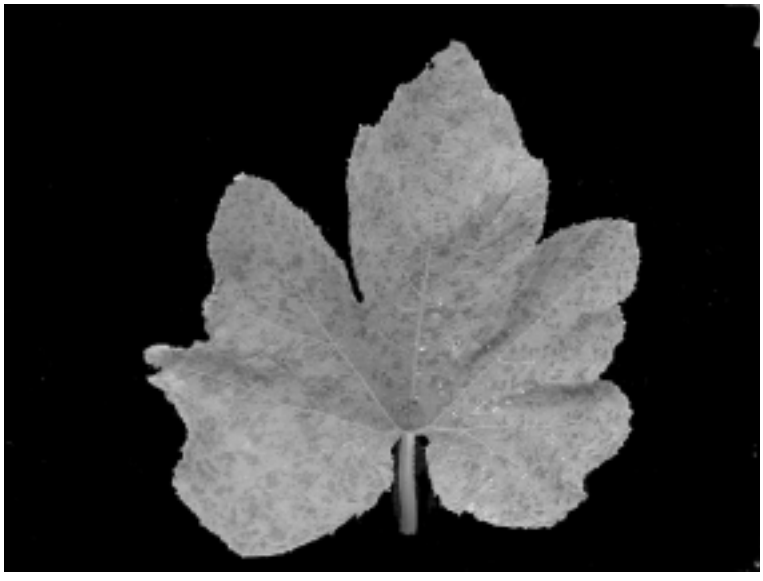

Fig. 3 — Chlorotic mottling of leaves (sample 21) caused by watermelon mosaic potyvirus 2

Fig. 4 — Yellowing and green veinbanding of leaves (sample 38) caused by zucchini yellow mosaic potyvirus