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BIOLOGICAL AND SEROLOGICAL CHARACTERIZATION OF VIRUSES OF SUMMER SQUASH CROPS IN YUGOSLAVIA

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Abstract: A survey on summer squash open field crops was carried out during 2000 and 2001 in order to identify the major viruses infecting these crops in different localities. Plants showed different types of symptoms: mild mosaic, chlorotic spotting, distinctive mosaic, blistering of leaf lamina, leaf yellowing, deformation of leaf lamina, knobbed fruits and stunting of plants. The symptoms were very variable but showed the viral nature of the investigated summer squash diseases. The collected samples were tested by bioassay and by two serological methods ELISA and EBIA using cucumber mosaic cucumovirus (CMV), zucchini yellow mosaic potyvirus (ZYMV), watermelon mosaic potyvirus 2 (WMV-2), zucchini yellow flack potyvirus (ZYFV), watermelon mosaic potyvirus 1 (WMV-1), squash mosaic comovirus (SqMV) and cucurbit aphid-borne yellows polerovirus (CABYV) polyclonal antisera. In all tested samples single or mixed infection with ZYMV, CMV and WMV-2 was detected. The most prevalent virus infecting summer squash was ZYMV. This is the first report of ZYMV, the most destructive virus infecting cucurbits, in Yugoslavia. It was also proven that the identified viruses are transmissible by *Aphis gossypii* in a non-persistent manner, but possible role of seed in virus transmission was not confirmed.

Key words: summer squash crop, plant viruses, zucchini yellow mosaic potyvirus, watermelon mosaic potyvirus 2, cucumber mosaic cucumovirus, bioassay, serology.

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Introduction

Cucurbit plants (cucumber, watermelon, melon and squash) are very sensitive to viral infection. Over 30 viruses have been reported to infect these plants (Zitter et al., 1996). The diseases they cause can be very destructive and are not easy to be controlled. Their incidence, severity and importance varies and depends on the growth area, virus involved and the complexity of infection. The most of described viruses cause very serious losses in cucurbit production, but 6 of them have been considered to be economically the most important. They are: 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) (Webb et al., 1965; Milne et al., 1969; Lovisolo, 1980; Nameth et al., 1986; Kyriakopoulou and Varveri, 1991; Dikova, 1995; Lisa et al., 1997; Gallitelli and Davino, 1998; Tobias and Tulipan, 2002).

Although reports from all over the world point out that viruses cause the largest number and the most destructive diseases of cucurbits, viruses of these crops have been poorly studied in Yugoslavia. No research has been done and there have been no reports on viruses infecting summer squash (*Cucurbita pepo* L.). A small number of previous reports in former Yugoslavia are related to viruses of watermelon (Stakić and Nikolić, 1966), some cultivated cucurbits (Pejčinovski, 1978), and cucumber (Tošić et al., 1996).

In Yugoslavia, the problem of virus infection of squash (*Cucurbita pepo*), attracted attention in the year 2000, with its incidence in dramatic proportion (Dukić et al., 2001) when a new syndrome of the disease was noticed on cucurbits which triggered etiologic investigation.

Material and Methods

Sample collecting

The field surveys were done in 2000, in the locality of Veliko Selo, near Belgrade where the production was completely destructed and Futog, vicinity of Novi Sad and 2001 in the localities of Slanci, Begaljica, and Grocka. Samples were collected from summer squash cv. Beogradska tikvica and hybrid Ezra F1 with prominent symptoms of virus infection on leaf and small fruit with distinctive deformation, with knobby growth or deep cracks and whole habitus. Collected samples were further investigated in order to confirm the viral nature of the disease and to identify the causal agent. Investigations were performed by bioassay and serologically.

Bioassay

Pathogenicity test and biological characterization were performed by mechanical inoculation. For this purpose, tissue of young leaves or fruit with typical symptoms were homogenized in cold 0.01M phosphate buffer saline (PBS) pH 7.0 in 1:1 ratio (w/v) with abrasive added. Bioassay was performed on the following test plants: *Tetragonia expansa*, *Gomphrena globosa*, *Chenopodium amaranticolor*, *C. quinoa*, *C. murale*, *Citrullus lanatus* cv. Crimsonsweet, *Cucumis melo* cv. Ananas and cv. Sezam, *Cucumis sativus* cv. Sunčani potok and cv. Pariski kornišon, *Cucurbita pepo* cv. Beogradska and hybrid Ezra F1, *Luffa cylindrica*, *Lagenaria* sp., *Cucurbita moshata*, *Nicotiana tabacum* cv. Samsun, *N. clevelandii*, *N. benthamiana*, *N. glutinosa*, *Physalis floridana*, *Petunia hybrida*, *Lycopersicon esculentum* cv. Saint Piere, *Capsicum annuum* cv. Kurtovska kapija, *Datura stramonium*, *Ranunculus ficaria*, *Ranunculus sardous*, *Phaseolus vulgaris* cv. Panonski gradištanac, *Lathyrus odoratus* and *Vigna sinensis*. Inoculated test plants were maintained under greenhouse conditions. Symptom development was observed periodically every two days up to one month after inoculation.

Serological tests

In order to identify the virus, causal agent of summer squash disease, two serological methods were also used in the investigations: EBIA (Western blot) and DAS ELISA.

EBIA was performed according to the procedure of O'Donell et al. (1982) modified by Hewish et al. (1986). Samples were prepared as described by Laemmli (1970) and polyclonal antisera against CMV, ZYMV and WMV-2 (Bioreba AG, Switzerland) were used at 1:1000 dilution, where goat-antirabbit antisera (Bio-Rad Lab., Richmond CA. USA) was used at 1:2500 dilution in skimmed milk. The reaction was rated as positive if lilac blue color occurred on nitrocellulose membrane.

In DAS ELISA procedure (Clark and Adams, 1977), except the above mentioned antisera against WMV-1, SqMV, zucchini yellow flack potyvirus (ZYFV) and cucurbit aphid-borne yellows polerovirus (CABYV) were used. Commercial kits of specific polyclonal antibody and alkaline phosphatase-labelled conjugate γ-globulin (Bioreba AG, Switzerland) were used at 1:1000 dilution in corresponding buffers. 1g of leaf or fruit tissue was homogenized in 5 ml of 0.02M PBS with 0.05% Tween 20 and 2 % polivinyl pyrrolidone MM 40 000 (Avgelis and Katis, 1989). Substrate p-nitro phenyl phosphate (1 mg/ml) was added to plates and reactions were allowed to progress for half an hour at room temperature and absorbance values were determined with a plate reader at 405nm. Absorbance equal or greater than twofold the average for healthy control samples in the same plate –experiment were regarded as positive.

Aphid transmission

During crop examination abundant aphid colonies were recorded on diseased plants. Aphid species were identified, and simultaneously transferred to young healthy summer squash plants and kept under greenhouse conditions. Also, originating from field aphid colonies, aviruliferous adults were raised on summer squash cv. Beogradska tikvica and cabbage leaves on which they were also kept for further investigations.

Viruliferous aphid colonies from diseased field plants were transferred to individual summer squash plants where they were allowed to feed for two minutes after which plants were sprayed with insecticide.

Aviruliferous aphid adults, after a period of three hours of starving, were given a two minute acquisition period on infected summer squash plants and then aphids were transferred to healthy plants to feed for 24 hours.

Five plants of summer squash cv. Beogradska tikvica in 2-3 true leaf stage were inoculated with five adult aphids for each treatment.

Seed transmission tests

Seed transmission tests were made by using 500 seeds of summer squash cv. Beogradska tikvica and 500 seeds of hybrid Ezra F1 from commercial lot used in field production.

Seeds were sown in pots filled with steam sterilized soil and kept in greenhouse conditions. Seedlings were checked for virus by recording symptoms up to one month after emergence. In cases where virus-like symptoms occurred inoculations on *Chenopodium quinoa*, *Cucurbita pepo* cv. Beogradska tikvica and hybrid Ezra F1, serological tests by ELISA with ZYMV, WMV-2, CMV antisera and grafting by transplantation on healthy plants were done.

Results and Discussion

Field symptoms

Survey of summer squash crops in the locality of Veliko Selo showed that a large number of plots, where hybrid Ezra F1 and summer squash cv. Beogradska tikvica were grown, were completely destroyed. Infected plants demonstrated typical and severe viral disease symptoms. Also, within a given cultivar, some field plants were severely distorted and others were only mildly affected.

On leaves, fruit and in the overall growth habit of the plants symptoms were very prominent and variable, depending upon the host, the affected plant parts, and developmental stage at which the plants became infected. Plants were stunted and bushy because of shortened internodes with small, distorted or no fruit at all.

Foliar symptoms ranged from mild mosaic, chlorotic spotting, distinctive mosaic, blistering of leaf lamina, leaf yellowing, through different deformation to leaf lamina narrowing. The most striking symptoms occurred on infected fruit where knobby overgrowth covered the fruit surface. Deformation and abnormal green coloration occurred on fruit. Such fruits were often small and severely distorted with proliferations or longitudinal cracks, which was especially present on fruit originating from Novi Sad. Plants infected by the time fruit set begins produced unmarketable fruit.

The same symptoms on summer squash were found in the survey of other localities (Slanci, Begaljica and Grocka) in 2001.

Described symptoms are grouped into several types (table 2.). In many cases the same infected plant showed different types of symptoms.

Test plants reaction

Samples collected during the years 2000 and 2001 have been infected with ZYMV, CMV and WMV-2, based on the reactions of test plants and host range. The reaction of test plants and the host range have shown that it is possible to differentiate mechanically transmittable viruses of summer squash (table 1).

Investigated isolates of certain viruses demonstrated no differences in host range, nor symptom type which they caused on tested plants.

CMV differs clearly from other two viruses, before all, based on the reaction of *Nicotiana* spp. plants (*N. tabacum*, *N. glutinosa*, *N. clevelandii*) where it causes mosaic, systemic type of symptoms, while ZYMV and WMV-2 are not infective for these plants. CMV is the only of three viruses infectious to *Physalis floridana*, *Petunia hybrida*, *Vigna sinensis*, *Lycopersicon esculentum*, *Capsicum annuum*. On *Luffa cylindrica* CMV causes characteristic mosaic symptoms, but WMV-2 causes latent infection. On *Citrullus lanatus* CMV, like ZYMV and WMV-2, causes mosaic, but in rare cases it also causes the appearance of large chlorotic local lesions.

Symptoms on test plants induced by our CMV isolates are the same as those reported by other authors (Lastra, 1968; Cohen and Nitzany, 1963; Tobias and Tulipan, 2002).

ZYMV and WMV-2 have a very similar host range, but differ in some cases. WMV-2 opposite to ZYMV, on *Tetragonia expansa*, *N. benthamiana* and *Luffa cylindrica* causes latent infections which were proven by back inoculations on *Chenopodium quinoa*. After inoculation with WMV-2, local chlorotic lesions followed by prominent mosaic and leaf deformations appear on *Chenopodium quinoa*. Also, WMV-2 on *Chenopodium amaranticolor* inoculated leaves causes local chlorotic lesions which become necrotic in a few days, where all tested ZYMV isolates cause local chlorotic lesions which become necrotic only after the plant decays.

T a b . 1.- Test plants reaction after mechanical inoculations with ZYMV, WMV-2 and CMV

Test plant	Symptoms[1]		
Family, species, cultivar	ZYMV	CMV	WMV-2
Aizoaceae			
Tetragonia expansa	-	-	-*
Amaranthaceae			
Gomphrena globosa	LLn	/	LLn
Chenopodiaceae			
Chenopodum amaranticolor	LLc	LLn	LLn
Chenopodium quinoa	LLc	LLn	LLc, SM, D
Chenopodium murale	LLc	/	LLc
Cucurbitaceae			
Citrulus lanatus cv. Crimsonsweet	SM	LLc, SM	SM
Cucumis melo cv. Ananas	SM, D	SM, D	SM, D
Cucumis melo cv. Sezam	SM	SM	SM
Cucumis sativus cv. Sunčani potok	SM	SM	SM
Cucumis sativus cv. Pariski kornišon	SM	SM	SM
Cucurbita pepo cv. Ezra	SM, D	SM, D	rLLc, SM, D
Cucurbita pepo cv. Beogradska	SM, D	SM, D	SM, D
Luffa cylindrica	-	SM	-*
Lagenaria sp.	SM	SM	SM
Cucurbita moshata	SM	SM	SM
Solanaceae			
Nicotiana tabacum cv. Samsun	-	SM	-
Nicotiana clevelandii	-	SM	-
Nicotiana benthamiana	-	/	-*
Nicotiana glutinosa	-	SM	-
Physalis floridana	-	SM	-
Petunia hybrida	-	SM	-
Lycopersicon esculentum cv. Saint Piere	-	SM, D, N	-
Capsicum annuum cv. Kurtovska kapija	-	SM	-
Datura stramonium	-	/	-
Ranunculaceae			
Ranunculus ficaria	-	-	-
Ranunculus sardous	-	-	-
Fabaceae			
Phaseolus vulgaris cv. Panonski gradištanac	-	/	-
Lathyrus odonatus	-	-	-
Vigna sinensis	-	LLc	-

[1] – = no symptom ; LLc = local lesions chlorotic; LLn = local lesions necrotic; SM = systemic mottling and mosaic; D = leaf deformation;
r = rare appearance of spots; / = not assessed
* latent infection

ZYMV isolates, obtained in our investigations, were not infectious to *Luffa cylindrica*, although the infectivity of this virus to *Luffa aegiptica* or *Luffa*

acutangula was proven for most isolates studied by other researchers (Lisa et al., 1981; Lisa and Lecoq, 1984; Provvidenti and Gonsalves, 1984; Prieto et al., 2001). Lesemann et al. (1983) described one ZYMV isolate from cucumber which was not infectious to *Luffa acutangula*, and that isolate is different from ours in its infectivity to *Nicotiana benthamiana*.

WMV-2 isolates investigated in the course of this study cause the same symptoms on most tested plants as those previously described (Provvidenti and Schroeder, 1970; Purcifull et al., 1984), although our isolates do not induce mosaic on *Nicotiana benthamiana*, but latent infection. This makes our isolates different from some described by other authors (Tošić et al., 1996; Tobias and Tulipan, 2002). Also, our WMV-2 isolates cause the appearance of numerous chlorotic spots on inoculated leaves of *C. quinoa*, as many described isolates, but they belong to a small number of isolates (Lisa and Dellavalle, 1981, Purcifull et al., 1984, Tošić et al., 1996) which can infect this plant systemically causing mosaic and leaf deformation.

Test plants symptoms as well as serological analysis confirmed that ZYMV, CMV and WMV-2 are present in single or in mixed infections. Out of 20 tested samples, 6 of them (30%) showed to be single virus infection, and in 9 (45%) cases the complex infections with two viruses were proven (table 2). In the remaining 5 (25%) samples complex infections with all three viruses were confirmed. ZYMV was detected in single or mixed infections in 15 (75%), CMV in 13 (65%), and WMV-2 in 9 (45%) samples.

Tab.2. - Viruses identified and their connection with symptom types on infected plants in the field

Locality	Cultivar/Hybrid (No. of samples)		Symptom type*	Virus (No. of samples)	
Futog	Beogradska	(2)	5	ZYMV[a,b,c]	(2)
Veliko Selo	Ezra F1	(6)	1, 2, 3, 5	ZYMV[a,b,c]	(1)
				WMV-2[a,b,c]	(1)
				ZYMV[a,b,c]+CMV[a,b,c]	(1)
				ZYMV[a,b,c]+CMV[a,b,c]+WMV-2[a,b,c]	(1)
				CMV[a]+WMV-2[a,b,c]	(2)
Begaljica	Beogradska	(4)	1, 3	ZYMV[a,b,c]	(2)
				ZYMV[a,b,c]+CMV[a,b,c]	(2)
Slanci	Beogradska	(2)	1, 3, 5	ZYMV[a,b,c]+CMV[a]	(1)
				ZYMV[a,b,c]+WMV-2[a,b,c]	(1)
	Ezra F1	(2)	1, 3, 5	ZYMV[a,b,c]+CMV[a]+WMV-2[a,b,c]	(2)
Grocka	Beogradska	(4)	1, 4	ZYMV[a,b,c]+CMV[a]	(2)
				ZYMV[a,b]+CMV[a]+WMV-2[a,b,c]	(2)

*symptom type: 1= plant stunting; 2= mild mosaic, chlorotic spotting;
3= distinctive mosaic, leaf deformation; 4= blistering, yellowing of leaves;
5= fruit deformation, knobby overgrowth or cracks
[a] virus identification by bioassay
[b] virus identification by ELISA
[c] virus identification by EBIA

Symptoms induced by ZYMV, CMV i WMV-2 on cucurbits in field (table 2) are various and there is no connection between the type of symptom and the virus involved, which suggests the impossibility of diagnosing the disease based on field symptoms.

Serological analysis

Results of virus identification obtained by bioassay were confirmed by serological tests (ELISA and EBIA) (table 2).

Polyclonal antisera against ZYMV and WMV-2 could detect viruses in all tested samples by both applied serological methods used. On the other hand, CMV specific antiserum reacted positively only with four out of 13 samples containing that virus. With the remaining samples, where that virus was proven by bioassay, no serological reaction was observed. Most probably the absence of serological reaction was due to low antigen concentration or the quality of commercial CMV antisera. Polyclonal antisera specific to ZYFV, WMV-1, SqMV and CABYV did not react positively with tested samples in ELISA test.

Results show that both serological methods are sensitive and suitable for serological testing of infected summer squash plants, although ELISA has shown to be more suitable for its worldwide routine use in summer squash virus infection testing (Menassa et al., 1986; Yuki et al., 2000), and because of its possibilities of testing a large number of samples simultaneously.

Aphid transmission

Transmission of the investigated viruses were attempted with the cotton aphid *Aphis gosypii* which was predominant insect in surveyed fields. Some adults of *Aphis gosypii,* found on infected summer squash plants under field conditions, were directly transferred to summer squash test plants for inoculation. All summer squash test plants infested with viruliferous aphids developed typical symptoms and the viruses were proven by back inoculations.

In that way it was proven that investigated ZYMV, CMV and WMV-2 isolates are nonpersistently transmissable by *Aphis gosypii*.

Seed transmission

Out of 1000 emerged tested seeds, only two summer squash seedlings cv. Beogradska tikvica, showed local chlorotic spots on cotyledon leaves which reminded of virus caused infections. None of the applied methods as bioassay, ELISA or grafting, did not confirm the viral nature of the symptoms which occurred on these two plants. The rest of the seedlings were symptomless up to two months after emerging.

Health check of seed lots used in commercial production did not show viral infection, suggesting that the seed has no role in the epidemiology of the studied summer squash diseases. These results are in agreement with those obtained by some researchers (Robinson et al., 1993; Orozco-Santos et al., 1994), while some researchers (Schrijnwerkers et al.,1991) pointed out transmissibility of ZYMV with summer squash seed, but in very low percentage. Tobias and Tulipan (2002) also point out that seed transmission of ZYMV can be achieved in a few promiles resulting in 7-8 infected plants per hectare, which provides a source of inoculum. Neither for WMV-2 (Stakić i Nikolić, 1966) nor for CMV (Zitter et al., 1996) seed transmission has been shown.

Conclusion

New disease syndrome of summer squash (*Cucurbita pepo*) cv. Beogradska tikvica and hybrid Ezra F1, appearing with prominent plant stunting, leaf yellowing, mosaic, blistering of leaf lamina, different leaf deformation with deformed fruit covered with knobby overgrowth, proliferations or longitudinal cracks occurred for the first time in the year of 2000, and repeated in 2001. In some localities the incidence of this disease completely destroyed or seriously endangered summer squash crops. Infected plants demonstrated typical and severe viral disease symptoms.

Etiologic investigations have shown that the disease is of a viral nature. Identification of the virus was done using bioassay and serological analysis. Biological characterization of the viruses was done on different indicator plants, in order to determine the host range and plants reaction. Samples were also tested by ELISA using polyclonal antisera against CMV (cucumber mosaic cucumovirus), ZYMV (zucchini yellow mosaic potyvirus), WMV-2 (watermelon mosaic potyvirus 2), ZYFV (zucchini yellow flack potyvirus), WMV-1 (watermelon mosaic potyvirus 1), SqMV (squash mosaic comovirus) and CABYV (cucurbit aphid-borne yellows polerovirus). Serological investigations by EBIA method were done using polyclonal antisera against CMV, ZYMV and WMV-2.

Based on symptomatology of test plants and confirmed by serological reactions, it is concluded that the investigated samples have been infected in single or mixed infections with ZYMV, CMV and WMV-2. ZYMV, which was reported for the first time in Yugoslavia, was identified in most samples.

Aphid colonies found on summer squash plants under field conditions were determined as *Aphis gossypii*. Transmission tests have shown that identified viruses are transmitted by that vector in a non-persistent manner.

Checking infectivity in seed lots used in commercial production of summer squash, did not show the possible role of seed in disease transmission.

Serious destructivity, viral nature, the presence of ZYMV new pathogen for Yugoslavia and new hosts for CMV and WMV-2 show the necessity for biological and molecular characterization of mentioned viruses and intensive studies of the epidemiology of summer squash virus diseases.

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BIOLOŠKA I SEROLOŠKA KARAKTERIZACIJA VIRUSA TIKVICA U JUGOSLAVIJI

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R e z i m e

U toku 2000. i 2001. godine izvršen je pregled useva tikvica za jelo da bi se identifikovali osnovni virusi infektivni za tikvice u različitim lokalitetima. Biljke su pokazivale različite simptome: blagi mozaik, hlorotičnu pegavost, izraženi mozaik, klobučavost liske, žućenje lista, deformacije liske, bradavičaste izraštaje na plodu i kržljavost biljaka. Simptomi su veoma varijabilni i na osnovu njih se ne može obaviti determinacija virusa prouzrokovača oboljenja. Sakupljeni uzorci su testirani biotestom, kao i sa dve serološke metode, ELISA i EBIA korišćenjem poliklonalnih antiseruma na cucumber mosaic cucumovirus (CMV), zucchini yellow mosaic potyvirus (ZYMV), watermelon mosaic potyvirus 2 (WMV-2), zucchini yellow flack potyvirus (ZYFV), watermelon mosaic potyvirus 1 (WMV-1), squash mosaic comovirus (SqMV) i cucurbit aphid-borne yellows polerovirus (CABYV). U svim ispitivanim uzorcima dokazana je pojedinačna ili mešana infekcija sa ZYMV, WMV-2 i CMV. Najčešće infekcije su bile sa ZYMV. Ovaj virus, jedan od najdestruktivnijih virusa na vrežastim kulturama, prvi put je konstatovan u našoj zemlji. Takođe je utvrđeno da se identifikovani virusi prenose na neperzistentan način vašima *Aphis gossypii*, a moguća uloga semena u pojavi oboljenja nije potvrđena.

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