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FIRST RECORD OF *TOMATO BLACK RING VIRUS* (TBRV) IN THE NATURAL INFECTION OF *CUCUMIS SATIVUS* IN POLAND

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Abstract: From the naturally infected cucumber plant spherical virus was isolated that mainly on the basis of its serological properties has been identified as *Tomato black ring virus* (TBRV). Using antiserum against TBRV-ED for the specific trapping of virus followed by PCR test (immunocapture-RT-PCR) allowed to distinguish TBRV from related viruses, especially *Beet ringspot virus* (BRSV). Presence of as many as two satellite RNAs should be found as a unique feature of the cucumber isolate.

Key words: cucumber, TBRV, serology, immunocapture RT-PCR, satellite RNA

INTRODUCTION

Tomato black ring virus (TBRV) has been noticed almost all over the world and naturally infected very wide range of monocotyledonous and dicotyledonous species. Sometimes the virus is able to decrease significantly the yield of plants, especially perennial crops. In Poland TBRV is classified as a quarantine pathogen. The virus is not noticed frequently in the country nevertheless, over last years has infected a number of species of crop plants such as potato (Chrzanowska and Śniegowski 1965), tomato (Twardowicz-Jakusz 1969; Pospieszny and Borodynko 1999), celery (Twardowicz-Jakusz 1976), carrot (Twardowicz-Jakusz et al. 1977a), horseradish (Twardowicz-Jakusz et al. 1977b), flox (Kamińska and Woś 1978), gladiolus (Kamińska and Woś 1978), forsythia (Kamińska and Sobiło 1983) as well as wild ones i.e. privet (Błaszczak and Pospieszny 1987) and black locust (Pospieszny and Borodynko 1999).

This work presents an identification of TBRV from naturally infected cucumber plant.

MATERIAL AND METHODS

Virus source and experimental host range

Tested virus was isolated from *Cucumis sativus* cultivated in a home garden. Plants showing symptoms of growth inhibition, slight deformation of leaves and green-yellowish spots on them were used for the experiment. TBRV isolates had been obtained formerly from crop plants but only that of potato (Chrzanowska and Śniegowski 1965) was available as a kind gift from Professor Chrzanowska of IHAR, Radzików, Poland. Both isolates were maintained under greenhouse conditions using *C. quinoa* as a host and afterwards reservoir of the virus for studies on its host range. Whole plants of *C. quinoa* showing systemic symptoms (6–9 days after inoculation) were ground with phosphate buffer at the ratio of 1:5 (w/v). Next the tested plants were inoculated with the obtained sap. The infected plants were kept in the greenhouse at 18–27°C and exposed to 14 hr light period. Reaction of plants to the virus infection was then observed every other day. Plants that did not show any symptoms were verified by reinoculation of *C. quinoa* with their sap.

Virus purification

Freshly harvested (or frozen) systemically infected *C. quinoa* plants were homogenized in 0.1 *M* citric buffer pH 7.2, containing 1% of Na_2SO_3.

The homogenate was filtrated through two layers of cheesecloth and the extract stirred for 1 min with 1.5 vol. of cold chloroform. The mixture was then centrifuged at 5000 *g* for 15 min and a clear aqueous layer was incubated for 1.5 hr at 4°C with PEG-6000 and NaCl at final concentrations of 6% (w/v) and 0.2 *M*, respectively. The precipitate was collected by centrifugation at 10000 *g* for 15 min and the pellet resuspended in the buffer. Clarified suspension was centrifuged at 120000 *g* for 2.5 hr at 4°C and the resulted pellet was resuspended in a few milliliters of the buffer. Supernatant after clarification was centrifuged in 10–40% sucrose density gradient. Purified virus collected from sucrose gradient was concentrated by high-speed centrifugation at 120000 *g* for 3 hr and clarified by low speed centrifugation at 5000 *g* for 10 min.

Electron microscopy

Virus particles from infected plants or purified preparations were stained with 2% phospotungstic acid, PTA (adjusted to pH 7.2) and examined by electron microscopy.

Serology

Studied virus was identified by immunoelectron microscopy (Milne and Luisoni 1977) using antisera against: *Arabis mosaic virus* (ArMV), TBRV, *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV), *Beet ringspot virus* (BRSV) and *Broad bean stain virus* (BBSV).

Antisera of 1:100 and 1:10 dilution were used for trapping and decoration, respectively.

Taking into consideration strong serological affinity of TBRV and BRSV (formerly TBRV-S serotype) further identification was performed using ELISA test. The test was carried out as described by Clark and Adams (1977).

Antisera for serotypes TBRV-ED and TBRV-S (currently BRSV) were a kind gift from Dr. Le Gall of INRA, France.

Immunocapture-RT-PCR

Leaves of infected *Ch. quinoa* were crashed by pestle in 2 ml tubes with 500 μl of ELISA buffer (PBS with 0.05% Tween-20; 2% of polyvinylopirolidon, PVP, MW=40000 and 0.2% of albumin) than centrifuged for 5 min at 10000 *g*. Fifty μl of supernatant was pipetted into 200 μl PCR-tubes precoated for 1 hr at 37°C with 1000 times diluted antibodies against TBRV-ED. The supernatant was incubated for 1 hr at 37°C and than washed 3 times with PBS-T (PBS with 0.05% Tween-20). Before RT-PCR reaction, to the PCR-tubes 6 μl of 1.7% Triton X-100 was added. For PCR amplification following primers (Le Gall et al. 1995) were used:
P1 (downstream) 5'-ATGGGAGAAGTGCTGG and
P2 (upstream) 5'-ATCTTTTTGTGTCCAAC.

The samples were vigorously shaken and than to each sample the RT-PCR mixture [1xPCR buffer, 0.2 m*M* of dNTP, 20 U of RNAse inhibitor, 0.4 μM of primer P1, 0.4 μM of primer P2, 100U of reverse transcriptase (M-MuLV) and 1.25U of Taq DNA polymerase] was added. The reaction was performed on Biometra termocycler applying following thermal profile: 42°C – 20 min., 94°C – 2 min., (94°C – 20 s, 53°C – 20 s, 72°C – 30 s) – 35 cycles, 72°C – 5 min., 4°C – hold. The RT-PCR product was tested on 1% agarose gel in 1xTBA buffer.

RNA analysis

Purified virions were incubated in the extraction buffer (50 m*M* Tris HCl, pH 7.5; 50 m*M* NaCl; 5 m*M* EDTA; 1% SDS; 1 mg ml^{-1} proteinase K) for 1hr at 37°C and then RNA extracted by addition of phenol/chloroform/isoamyl alcohol (24:24:1, v/v) mixture and vigorous shaking. Next, the samples were centrifuged for 5 min at 10000 *g*. Upper layer was collected and phenol extraction repeated. Then upper layer was extracted twice with chloroform/isoamyl alcohol. RNA was precipitated for 30 min at -80°C with 96% ethanol containing 0.1 vol. 3 *M* sodium acetate, pH 4. The pellet was washed with 70% ethanol and dried using speed vac. Pellet was dissolved in 20 ml of RNase-free water and 2 ml sample of purified RNA dissolved in FORMAzol (MRC) and separated using 1% agarose gel.

RESULTS

Experimental host range and symptoms

Range of plants infected with the virus via mechanical inoculation and types of occurred symptoms are presented in table 1.

The virus infected numerous plant species of *Solanaceae* family, except *Capsicum annuum*. Pathogen caused repeatable on the leaves of *Nicotiana tabacum*, cvs. Xanthi nc. and Samsun characteristic rings and pattern lines. Nevertheless, other species like for example *N. glutinosa* and *N. affinis* reacted rarely with local rings but rather showed chlorotic spots. Systemic infection in most of tobacco species occurred in the beginning as an acute mosaic followed by necrotic spots and deformation of leaves but those symptoms did not appear on new leaves. Systemic symptoms char-

Table 1. Experimental host range for TBRV from cucumber

Hosts	Symptoms
Nicotiana tabacum cv. Samsun	Lrs; Sm, Schs, Sd, R
N. tabacum cv. Xanthi nc	Lrs; Sm, Schs, Sd, R
N. tabacum cv. White Burley	Lchs,; Srs, Sm, Sd, R
N. affinis	Lchs; Srs, Sm, R
N. glutinosa	Lchs, Lrs, Schs, Sm, R
N. benthamiana	-, s
Gomphrena globosa	-' s
Petunia hybrida	Lch-ns; (Sm),s
Lycopersicon esculentum	-; s, Sn
Nicandra physaloides	(Lchs); s
Physalis floridana	(Lchs); Sm, Sns
Capsicum annuum	-; -
Chenopodium quinoa	Ln-chs; Sm, Sd
C. amaranticolor	Ln-chs; Sm
C. murale	Ln-chs; Sn
C. album	Lns; Sm, Sn
C. ficifolium	(Lch); Sch-ns, Sm
Spinacia oleracea	-; Sm (s)
Beta vulgaris	-, s
Phaseolus vulgaris	(Lchs); Sn, Sd, Sm
Pisum sativum	-; Sns, Sn, Sm, Sd
Vicia faba	-
Lupinus luteus	-
Lactuca sativa	-; s
Tetragonia expansa	Lchs; Sm, (s)
Cucumis sativus	Lchs; Sm
Antirrhinum maius	-;-
Ammi maius	-; Sm
Heliantus sativus	-; s
Brassica rapa	-
Sinapis alba	-; s, (Sm)
Zinnia elegans	-; s
Amaranthus retroflexus	Lns; Sm

Lchs= local chlorotic spots, Lrs= local ring spots, Lch-ns= local chlorotic-necrotic spots, Lns= local necrotic spots, Sm= systemic mosaic, Schs= systemic chlorotic spots, Sd= systemic deformation, R= recovery, Srs= systemic ring spots, Sns= systemic necrotic spots, Sn= necrosis of top, s= symptomless infection, ()= symptoms appeared sporadically, -=no symptoms, no virus.

acteristic for pea and bean plants infected with the virus were necrotic spots of leaves and necrosis of plant tops. On the other hand, when the virus had been applied in low concentration, only mosaic as the symptom was observed. Similar correlation was noticed for other plant species. The virus often strongly infected plants of *Chenopodiaceae* family causing their frequent decease.

Following inoculated plants species were not infected: *C. annuum*, *Vicia faba*, *Lupinus luteus*, *Brassica rapa* and *Antirrhinum maius*.

Electron microscopy and purification

In the sap from *C. quinoa* infected with the virus in electron microscope virus particles of ca. 30 nm in diameter were observed. Sometimes virions organized in the

tubular structures were noticed. During centrifugation of the purified virus preparation in sucrose gradient virus particles sedimented as three opalescent zones in transmitted light. Top zone was of low intensity, while middle and bottom ones of similar volumes were very well visible. Observations of the preparation in electron microscope confirmed a presence of not numerous, empty protein shells that appeared in top zone (Fig. 1).

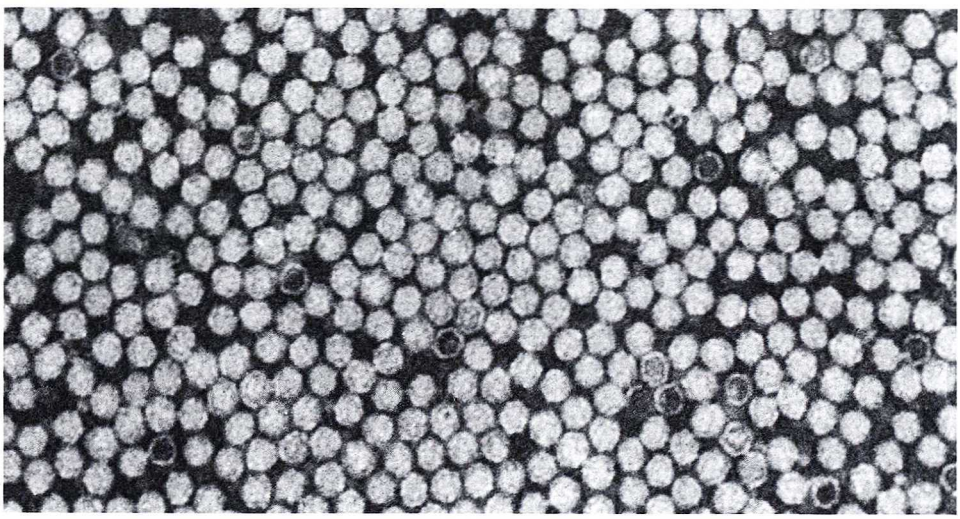

Fig. 1. Purified preparation of *Tomato black ring virus* from cucumber

Serological identification

In electron microscopy decoration test the virus was strongly decorated with antiserum against TBRV-ED and much weaker with TBRV-S (currently BRSV) one (Fig. 2). In ELISA test performed with antisera TBRV-ED and TBRV-S only the first one gave a positive reaction.

Immunocapture-RT-PCR

The primers used for IC-RT-PCR were designed (Le Gall et al. 1995) on the basis of 3'UTR sequence of BRSV and appeared as universal for detection of TBRV and close related to nepovirus like *Grapevin chrome mosaic virus* (GCMV). Those three viruses were characterized by distinct differences in serological properties. Above features were used for increasing of RT-PCR-detection specificity. The antibodies against TBRV-ED were used for trapping of TBRV particle from tested plants. The results of IC-RT-PCR for TBRV-cucumber and TBRV-potato were 300-nucleotides products, typical for most TBRV isolates (Fig. 3).

RNA analysis

The electrophoresis of cucumber isolate RNA showed an expected size of genomic RNAs (about 7.4kb and 4.6kb) and appearance of two additional, small satRNAs of size of ca. 500 and 1200 nucleotides (Fig. 4). Potato isolate of TBRV did not contain any sat RNA.

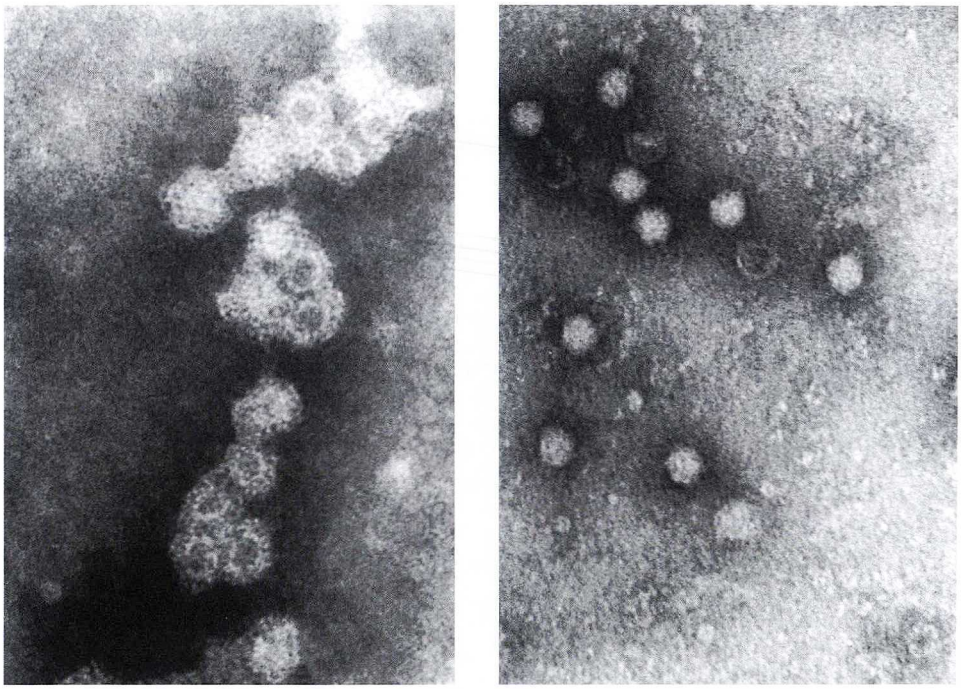

Fig. 2. Electron microscopy of *Tomato black ring virus* particles decorated with TBRV antiserum (left) and not decorated (right)

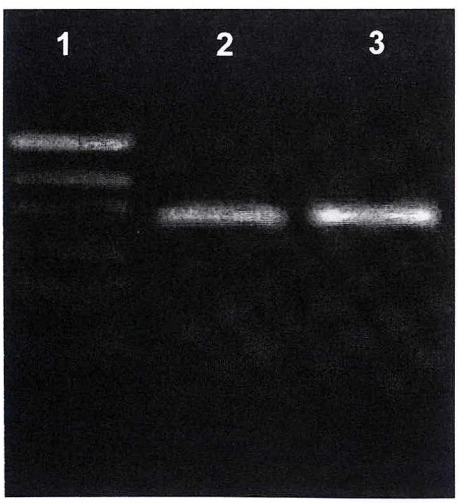

Fig. 3. Electrophoretic mobility of IC-RT-PCR product
Lane 1. DNA leader pUC19DNA/Mspl, (501, 404, 331, 242, 190, 147, 111, 67, 34)
Lane 2. TBRV-cucumber
Lane 3. TBRV-potato

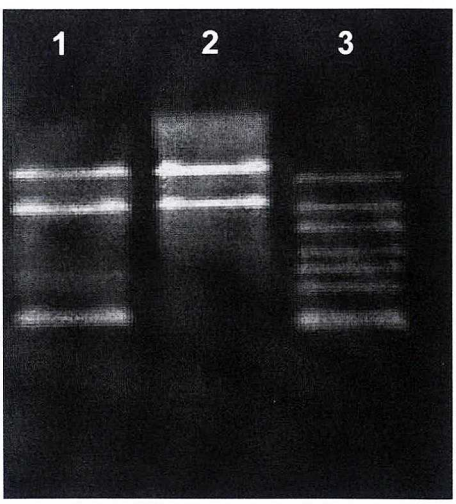

Fig. 4. Electrophoretic separation of viral RNA on 1% agarose gel
Lane 1. TBRV-cucumber
Lane 2. TBRV-potato
Lane 3. RNA leader (6kb, 4 kb, 3 kb, 1,5 kb, 1 kb, 0.5 kb, 0.2 kb)

DISCUSSION

The main aim of this work was an identification of the virus that occurred in the naturally infected cucumber plants. Results obtained from mechanically inoculated plants seem to be of low usefulness because they have been characteristic for numerous virus species of *Nepovirus* genus. Moreover, symptoms of disease are not repeatable during the seasons and depend on environmental conditions so, often disappear. However, an accurate biological characteristic may be useful for differentiation of the isolates of the virus existing in a number of variants. Observations performed by electron microscopy and characteristics of purified virus preparation support an information that allows for classification of the virus but at best on the genus level, i.e. *Nepovirus*. The opportunities for nepoviruses identification resulted from low serological affinity or lack of it among most virus species. Therefore, serological tests followed by IC-RT-PCR clearly showed that tested virus was identical to TBRV. In Poland this virus is represented by a number of isolates being biologically different but serologically similar (Borodynko et al. 2001). Apart from biological properties other ones can be applied for differentiation of TBRV isolates, e.g. presence and size of satellite RNAs. TBRV isolated from naturally infected cucumber contained two of them. Some satellite RNAs had been found before in Polish isolates of the virus from other plants nevertheless, presence of as many as two of them is the first case.

Presented paper is the first report describing an occurrence of TBRV in the naturally infected *Cucumis sativus* in Poland.

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POLISH SUMMARY

PIERWSZE DONIESIENIE O WYSTĘPOWANIU WIRUSA CZARNEJ PIERŚCIENIOWEJ PLAMISTOŚCI POMIDORA (*TOMATO BLACK RING VIRUS*) W NATURALNEJ INFEKCJI OGÓRKA W POLSCE

Z naturalnie porażonej rośliny ogórka wyizolowano po raz pierwszy izometrycznego wirusa, którego na podstawie głównie jego właściwości serologicznych zidentyfikowano jako wirus czarnej pierścieniowej plamistości pomidora (*Tomato black ring virus*, TBRV). Zastosowanie surowicy na serotyp TBRV-ED do specyficznego wyłapywania wirusów poprzedzającego test reakcji łańcuchowej polimerazy (immunocapture-RT-PCR) pozwoliło na jednoznaczne odróżnienie TBRV od wirusów pokrewnych a szczególnie wirusa pierścieniowej plamistości buraka (*Beet ringspot virus*, BRSV). Za unikalną właściwość ogórkowego izolatu TBRV należy uznać obecność aż dwóch satelitarnych RNA.