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Isolation and characterization of *tobacco necrosis virus* detected on some vegetable species

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A virus disease agent was isolated from greenhouse-grown cucumber (*Cucumis sativus* L.), field-grown common bean (*Phaseolus vulgaris* L.) and white cabbage (*Brassica oleracea* var. *capitata* (L.) Alef) crops in Lithuania. The diseased vegetable plants expressed various spotting or mottling symptoms on leaves or fruits and necrotic symptoms on older leaves. The objective of this study was to identify the causal agent of the virus-like disease of these plant species. For the identification of the causal virus, the infection from cucumber, common bean and white cabbage leaves or fruits was transmitted to main test plant species which reacted by only local lesions. In electron microscopic preparations from infected plants, only icosahedral virus particles about 26 nm in diameter were detected. According to particle morphology, host range and symptoms, the causal virus was identified as *tobacco necrosis virus* (TNV). This finding was supported by the reverse transcription–polymerase chain reaction (RT–PCR) technique which confirmed that the virus isolates from the Lithuanian cucumber, common bean and white cabbage crops were identical with the TNV strain A "Nebraska" isolate.

Key words: cabbage, cucumber, bean, *tobacco necrosis virus,* identification, EM, RT-PCR

INTRODUCTION

Cucumber (*Cucumis sativus* L.), common bean (*Phaseolus vulgaris* L.) and white cabbage (*Brassica oleracea* var. capitata (L.) Alef) are natural hosts of numerous widespread viruses [1, 2] which greatly reduce yield quality. These plants are potential natural alternative hosts of other viruses, including *arabis mosaic virus, tomato ringspot virus* (ToRSV) and *tobacco necrosis virus* (TNV) [2, 3]. *Cucumber mosaic virus*, *cucumber green mottle mosaic virus* and ToRSV in Lithuania have been isolated from cucumber [4], *bean common mosaic virus* and *bean yellow mosaic virus* from common bean [5], *cauliflower mosaic virus* and *turnip mosaic virus* from cabbage [6].

TNV, a member of the genus *Necrovirus,* was detected for the first time in greenhouse-grown tobacco seedlings in Japan and from root extracts was transmitted to herbaceous indicator plants [7]. Symptoms caused by TNV consist in local lesions that may vary in colour and size. The virus occurs in plant roots and can be transmitted mechanically to plant leaves. TNV is a frequent cause of cucumber necrosis in the European area. Numerous grey-brown spots occur in infected leaves, the interveinal tissue becomes necrotic and drops out [2]. TNV was isolated from greenhouse zucchini plants in Northern Italy, showing yellow spots on young leaves and necrotic symptoms on older leaves, petioles and stems [8]. TNV causes stipple streaks on bean leaves [2, 9]. TNV was detected in tobacco, tulip, bean, soybean, cucumber, lettuce, potato, some woody plants such as pear, apple, citruses, grapevines and olive [7, 10, 11]. TNV can infect 298 species in 167 genera of 54 families [12]. Numerous TNV strains have been grouped into two species: TNV–A and TNV–D. The virus survives in unsterilized greenhouse soil and can be spread by zoospores of the fungus *Olpidium brassicae* (Wor.) Dang and soil water. TNVs are highly stable small icosahedral particles 26–28 nm in diameter, containing single-stranded positive–sense RNA [1, 7, 10]. In Lithuania, TNV was detected in plum trees, strawberry, raspberry [13, 14] and ornamental plants [15].

The objectives of this study were to determine the incidence of the virus disease in cucumber, white cabbage and common bean crops in Lithuania, and to identify the causal agent, basing on the test plant symptoms, the morphology of virions and the size of PCR product.

MATERIALS AND METHODS

Samples of vegetable leaves or fruits were collected in greenhouses and fields from Vilnius, Radviliškis and Kėdainiai districts. Cucumber, common bean and white cabbage plants

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with pronounced visual virus-like symptoms such as dark-green-yellowish spots with bright mottling and necrotic vein-like pattern were investigated for virus presence.

The experimental work was carried out in the greenhouse and at Laboratory of Plant Viruses. The virus was identified by test-plant reaction to inoculation with isolates under study according to [1, 7, 10]. In order to differentiate virus isolates from diseased plant samples and identify the virus, test plants of *Aizoaceae* Rudolphi, *Amaranthaceae* Juss., *Chenopodiaceae* Vent., *Cucurbitaceae* Juss., *Fabaceae* Lindl and *Solanaceae* Juss. families were used.

Specimens for mechanical inoculation were prepared by homogenising the tissue of affected bean, cucumber, cabbage samples in 0.1 M sodium phosphate buffer, pH 7.0–7.2, containing as stabilising agents 0.02% 2-mercapthoethanol or 0.01 M sodium diethyldithiocarbamate (Na DIECA). Inoculations were performed with the aid of carborundum powder as an abrasive [16].

Presence of virus particles and their morphology were determined by investigating dip preparations negatively stained with 3% uranyl acetate in bidistiled water, using a transmission electron microscope (JEOL JEM-100S) at instrumental magnification of 25000 [16, 17].

Reverse transcriptase–polymerase chain reaction (RT–PCR) was used for the molecular detection of the virus isolated from vegetable samples and indicating similarity with TNV [18, 19]. Plant material, refrigerated at –20 °C or dessicated by lyophilization, was used in RT–PCRs.

RNA extraction was carried out according to the instruction of the "QuickPrep™ Total RNA Extraction Kit for the direct isolation of total RNA from most eukaryotic tissues or cells" (Amersham Biosciences, UK). Frozen tissue samples were ground in liquid nitrogen and transferred to microcentrifuge tubes; 150 µl of the extraction buffer was poured into a tube and 3 µl of 14.3 M 2-mercaptoethanol was added; 350 µl of lithium chloride (LiCl) and 500 µl of caesium trifluoracetate (CsTFA) solutions were added to the homogenized samples. The RNA formed a pellet at the bottom of the microtubes. The protein coat and the liquid phase were carefully removed, and washing of the total RNA pellets with three "Kit" components was carried out. The supernatants were discarded without disturbing the pellets; 1 ml of 70% ethanol was added to the samples. Diethyl pyrocarbonate (DEPC) treated water containing 1 µl of an RNAse inhibitor was added to the RNA pellets.

The primer pair used in RT–PCRs was designed for TNV-A "Nebraska" isolate: Fneb 5' (5'-ACA ATA GTC TCC AAC TCG GAG-3'), (910–930 nt) and RNeb 3' (5'-ATC ATA ACC TGC GTA AGG-3'), (1192–1209 nt). The viral sequence information is in the GenBank, Accession No. L04261.

Total RNA, resuspended from pellets in the solution containing RNAse inhibitor, primer RNeb and deionized water and incubated at 70 °C for 10 min, was used for DNA first-strand synthesis. This RNA solution was added to a mixture containing 5× reaction buffer, RNAse inhibitor, 2 mM dNTP mixture and RevertAid™ M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania). The synthesis of DNA first strand was carried out at 37 °C for 60 min and at 70 °C for 10 min. For DNA amplification reaction, mixtures containing 2 mM of dNTP mixture, both primers, 10 × PCR buffer with $MgCl_2$ and recombinant *Taq* polymerase (MBI Fermentas) were prepared. PCRs were carried out in an Eppendorf Mastercycler Personal for 40 cycles using the following parameters: 1 min at 94 °C (4 min for the first cycle), 2 min at 52 °C and primer extension for 2 min at 72 °C (10 min in the final cycle). PCRs products were analysed by electrophoresis in 5% polyacrylamide gel, stained with ethidium bromide, and DNA bands were visualized using a UV transilluminator. The fragment size standard of DNA was Φ × 174 DNA / *BsuRI* (*Hae*III) digest (MBI Fermentas) (from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp).

RESULTS AND DISCUSSION

Diseased samples of common bean, cucumber and white cabbage plants were observed and collected in commercial greenhouses and private fields in Vilnius, Kėdainiai and Radviliškis districts. Naturally infected plants showed dark-green and yellow spots on young leaves, and vein clearing and necrotic spots on older leaves. Their growth and fruiting were slightly reduced. Cucumber fruits were small, deformed, necrotic or yellow-mottled and severely puckered (Fig. 1). Symptoms of the virus in white cabbage leaves – vein clearing, mild mottling and plant stunting. In older leaves, these symptoms develop into yellow or brownish spots surrounded by irregular necrotic flecks (Fig. 2). Naturally infected common bean plants showed leaf distortion and mottling, followed by necrotic spots (Fig. 3) and stunted growth. Brown flecks appear on bean pods.

Fig. 1. Cucumber fruits affected by TNV

Fig. 2. TNV-induced symptoms on white cabbage leaf

Fig. 3. Distortion and mottling of bean leaves

For causal agent identification, all test plants were inoculated with extracts of leaf and fruit samples from diseased vegetables. The results of the host range study and the reaction of the main test plant species are summarized in Table. The isolates of the virus always induced only local necrotic, brown or chlorotic, white lesions on mechanically inoculated leaves of test plants. Symptoms of this virus were very characteristic on inoculated leaves of *Atriplex hortensis* L., *Gomphrena globosa* L., *Phaseolus vulgaris* cv. 'Bataaf', 'Red Kidney', 'Zlata Saxa', *Chenopodium* L. and *Nicotiana* L. species.

Table. **Reaction of main test plants to the causal agent isolated from vegetable crops**

Test plant	Symptoms
Amaranthus caudatus L.	N LL
Atriplex hortensis L.	N LL
Capsicum annuum L. 'Kristal'	0
Celosia argentea f. *Cristata* (L.) Kuntze	N LL
Chenopodium amaranticolor Coste et Reyn	Chl LL
C. ambrosioides L.	Br LL
C. foetidum Schrad.	N LL
C. quinoa Willd	Chl LL
C. urbicum L.	N LL
Cucumis sativus L. 'National pickling'	N LL
C. sativus L. 'Straight Eight'	Chl LL
Datura stramonium L.	0
Gomphrena globosa L.	Wh or N LL
Nicotiana alata Link et Otto	N LL
N. rustica L.	N LL
N. sylvestris Speg. and Comes	N LL
N. tabacum L. 'Samsun'	N LL
Phaseolus vulgaris L. 'Bataaf'	N LL; W, St
P. vulgaris L. 'Zlata Saxa'	N LL
P. vulgaris L. 'Red Kidney'	N LL
Tetragonia expansa Murr.	Chl LL

Notes: L – local reaction, N LL – necrotic local lesions, Chl LL – chlorotic local lesions, Br –brown, Wh – white, W – wilting, St – stunting, 0 – no reaction.

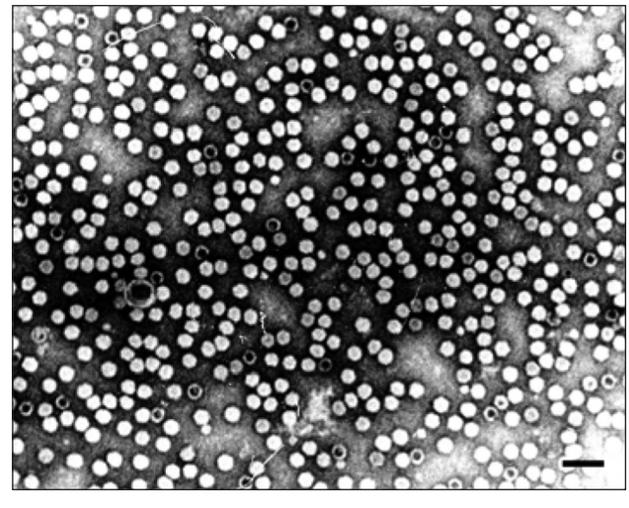

Fig. 4. Electronomicrograph of TNV particles. Bar represents 100 nm

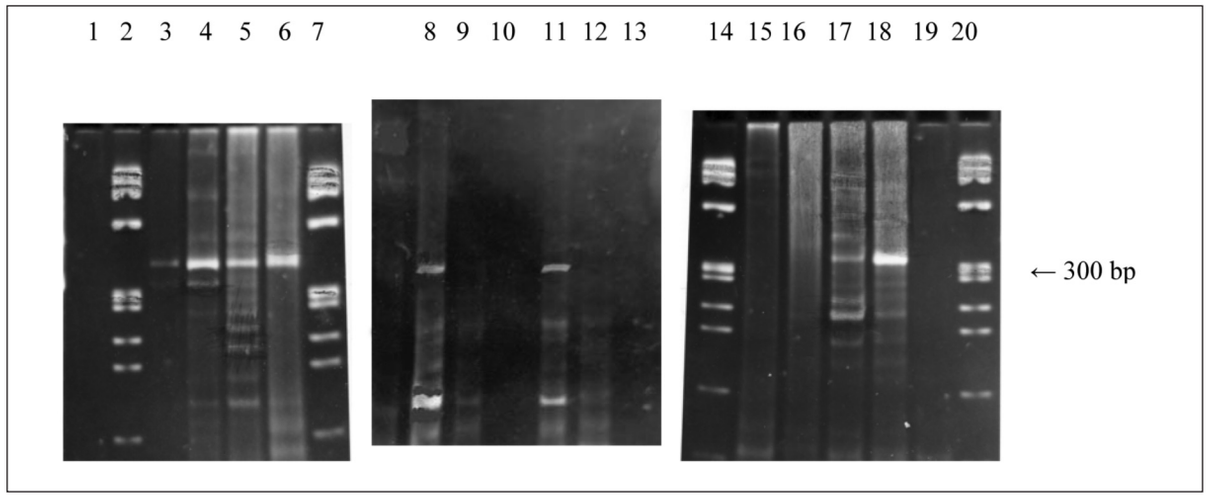

Fig. 5. cDNA products amplified in RT-PCRs from plants infected by TNV: 3–6 – plant samples with TNV from cucumber; 8 and 11 – TNV from bean; 17 and 18 – TNV from cabbage; 9, 10, and 19 – other viruses; 2, 7, 14, 20 – DNA ladder (from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp); 1, 12, 15 – water control; 13 and 16 – healthy plant samples

The inoculated common bean cv. 'Bataaf' sometimes indicated a drastic necrotic reaction which was followed by wilting of leaves and stunting of plants. No reaction was obtained on *Capsicum annuum* L. and *Datura stramonium* L. test plants inoculated with the virus isolates from the vegetable samples studied.

Icosahedral virus particles (about 26 nm in diameter) as characterictic of TNV were readily visualized by the EM in crude sap preparations from naturally infected cabbage, cucumber and bean samples and from mechanically inoculated various test plants (Fig. 4). The morphology of such particles in EM preparations was characteristic of *Necroviruses,* TNV including [7, 10].

The primer pair, designed for the TNV strain A "Nebraska" isolate on the basis of published sequences, specifically amplified copy DNA templates in RT–PCRs of virus isolates from cabbage, cucumber and bean crops (Fig. 5). Specific PCR products were obtained from experimentally infected samples of *P. vulgaris, N. rustica* and *A. hortensis* plants in isolates from cucumber, from samples of *N. rustica* and *Chenopodium amaranticolor* Coste et Reyn plants in isolates from white cabbage and from samples of naturally and experimentally infected beans. Specific bands were observed in the 5% acrylamide gel analysis at the position corresponding to the expected size of the copy DNA amplification products of about 300 bp. No PCR products were obtained in samples with negative controls (PCR mixture (without RNR) + PCR water, healthy plant tissues, or samples with other viruses). RT–PCR data confirmed the results of TNV identification by investigating the host range, symptoms and virus morphology.

This recently discovered virus of cucumber, cabbage and bean is already widely spread in many countries [1, 2]. The experimental host range and specific symptoms on all test plants, particles size and morphology indicate that the virus from vegetables most closely corresponds to the *tobacco necrosis virus,* a member of the genus *Necrovirus* [10]. RT–PCR data indicate that isolates from cucumber, cabbage and bean have a close similarity with the "Nebraska" isolate of TNV. Using a specific primer pair designed for D strain of TNV, no products of copy DNA amplification were detected. The "Nebraska" isolate has intermediate characteristics between strains A and D, although it is serologically related to both strains [20]. The RT–PCR product size of TNV isolates from vegetables showed an identity with TNV isolates identified in plums, strawberry, raspberry and ornamental plants [13–15].

Thus, results of TNV identification in some vegetables provide new information about the distribution of TNV in greenhouse-grown cucumber, field-grown white cabbage and common bean crops in Lithuania.

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KAI KURIOSE DARŽOVIŲ RŪŠYSE APTIKTO TABAKO NEKROZĖS VIRUSO IZOLIAVIMAS IR APIBŪDINIMAS

Santrauka

Daržovės (agurkai, baltagūžiai kopūstai, daržinės pupelės) su virusiniams pažeidimams būdingais simptomais buvo aptiktos komerciniuose šiltnamiuose ir privačiuose daržuose Vilniaus, Radviliškio ir Kėdainių rajonuose. Virusinės ligos pasireiškė tamsiai žaliomis ar gelsvomis dėmėmis ant jaunų lapų, gyslų nekroze, nekrozinėmis dėmėmis ant senų lapų ir lapų deformacija. Agurkų vaisiai buvo deformuoti, margi ir pūslėti, o pupelių ankštys – su nekroziniais įdubimais. Pažeisti kopūstai buvo smulkesni, jų lapai margi, su ryškiomis gyslomis. Sergančių daržovių lapų ar vaisių bei eksperimentiškai užkrėstų augalų ekstraktuose EM tyrimais aptikti nekrovirusams būdingi ikosaedriniai virionai. Pagal platų pažeidžiamų augalų spektrą, vietines nekrozes inokuliuotų augalų lapuose, virionų morfologines savybes (apie 26 nm skersmens) iš daržovių išskirti viruso izoliatai buvo identifikuoti kaip tabako nekrozės virusas (*tobacco necrosis necrovirus*). Panaudojus paskelbtus specifinius pradmenis ir molekulinį kopijinės DNR amplifikacijos atvirkštinės transkriptazės-polimerazės grandininės reakcijos (AT-PGR) testą buvo patvirtinta TNV A kamieno „Nebraska“ izoliato infekcija tirtose daržovių rūšyse Lietuvoje.

Raktažodžiai: agurkai, kopūstai, pupelės, tabako nekrozės virusas, identifikavimas, EM, AT-PGR