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Identification of nepoviruses in tomato (*Lycopersicon esculentum* Mill.)

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Abstract

A few nepoviruses (*Arabis mosaic nepovirus*, *Tobacco ringspot nepovirus* and *Tomato ringspot nepovirus*) have already been detected and identified in ornamental plants in Lithuania. The current paper presents the data on identification of two nepoviruses – *Tobacco ringspot nepovirus* (TRSV) and *Tomato black ring nepovirus* (TBRV) in tomatoes. Infected tomato plants were assayed by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) tests. Extracts of plants that showed positive reaction in DAS-ELISA were inoculated to more than twenty different test-plants. Local lesions and systemic infection were observed in plants belonging to *Amaranthus*, *Nicotiana*, *Chenopodium*, *Datura*, *Gomphrena* and *Tetragonia* genera. Transmission electron microscopy (EM) of extracts from leaves of infected test-plants revealed presence of icosahedral virus particles of ~30 nm in diameter that are characteristic of nepoviruses. Presence of TRSV and TBRV in infected test-plants was also detected by reverse transcription-polymerase chain reaction (RT-PCR) using virus-specific primers. Thus, detection and identification of TRSV and TBRV in naturally infected tomatoes and test-plants was confirmed by DAS-ELISA, EM and RT-PCR.

Key words: *Tobacco ringspot nepovirus*, *Tomato black ring nepovirus*, RT-PCR, identification.

Introduction

The nepoviruses (nematode-transmitted, polyhedral viruses) are a group of about 46 viruses that infect many plant families. They cause diseases of economic importance in a wide range of cultivated annual, perennial and woody plants, and are of serious concern to quarantine authorities worldwide. Their wide host range combined with ability to be transmitted by nematodes, seed and/or pollen makes them particularly dangerous and hard to eradicate and control (Murant, 1981; Brunt et al., 1996; Card et al., 2007). All nepoviruses have bipartite genome consisting of two linear single-stranded positive sense RNA species, both of which are transcribed as polyproteins. RNA-1 is ~8000 nucleotides in length and encodes the helicase, polymerase and proteinase. RNA-2 is ~4000–7000 nucleotides in length and encodes the capsid and movement proteins. Both RNA molecules have their 3' ends polyadenylated and virus specific peptides linked to their 5' ends. RNA-1 and RNA-2 are encapsidated separately. Nepovirus particles are isometric, polyhedral, ~30 nm in diameter. They are composed of 60 copies of single coat protein species and sediment as three components. All nepoviruses are transmitted through soil by free-living nematodes of *Longidorus* and *Xiphinema* species, feeding on roots. Members of the genus are categorised into three subgroups, A (8 species), B (9 species) and C (15 species), according to their serological relationships, sequence similarities, and the length and arrangement of RNA-2 (Harrison, Murant, 1977; Samuitienė, Navalinskienė, 2001; Samuitienė et al., 2003; Fauquet et al., 2005).

Tomato black ring nepovirus (TBRV) infects a wide range of economically important crop species, including grapevine, cherry, apricot, peach, berry-fruits (raspberry, currant, strawberry, blueberry), solanaceous species (potato, tomato, pepper, tobacco), and a number of weed and ornamental species (Harrison, Murant, 1977; Brunt et al., 1996; Edwardson, Christie, 1997). Symptoms, caused by TBRV, include systemic chlorotic or necrotic ringspot, leaf mottle and deformation; vein yellowing and stunting as well as necrotic spotting and flecking (Brunt et al., 1996). Economic losses for this virus are difficult to quantify as it is mild to asymptomatic in some species such as sugar beet (*Beta vulgaris* L.) and strawberry (*Fragaria sp.*), while causing yield losses of up to 40% on artichoke (*Cynara cardunculus* L.) (Harper et al., 2011). TBRV is transmitted both through seed and by the nematodes *Longidorus elongatus* and *Longidorus attenuatus* (Harrison et al., 1961; Brown et al., 1989). The virus has been reported in Europe, North and South America, India and Japan (Brunt et al., 1996; Harper et al., 2011). TBRV remains, however, a regulated pest in the North America, Australia, New Zealand and countries of the European Union, including Lithuania.

Tobacco ringspot nepovirus (TRSV) also occurs in a wide range of herbaceous and woody hosts. It causes significant disease in grape, soybean, tobacco, blueberry

and members of *Cucurbitaceae* family (melon, cucumber, squash, pumpkin). Many other hosts have been found naturally infected, including: apple, egg plant, blackberry, pepper, cherry, papaya and various weeds (Brown et al., 1989; Jossey, Babadoost, 2006). TRSV causes systemic chlorotic or necrotic ringspot, leaf deformation and stunting. The virus is transmitted by nematodes of *Xiphinema* family and through seeds (Harrison, Murant, 1977; Wang, Gergerich, 1998; Fuchs, 2009). TRSV has been reported in Europe, North America, Australia, Africa, India, Japan and New Zealand (Brunt et al., 1996; Ward et al., 2009). It is listed as quarantined pest in EPPO (1997) lists.

TBRV has not been previously reported in Lithuania. TRSV has already been found infecting ornamental plants (Samuitienė, Navalinskienė, 2001). However, in this paper, we report TRSV for the first time naturally infecting tomato in Lithuania.

Materials and methods

Virus isolates and test-plant method. Material for investigation was collected from private greenhouses growing tomatoes. In 2010–2011, experimental work was carried out at the greenhouse and Phytovirus Laboratory of the Institute of Botany of Nature Research Centre. Viruses have been identified by electron microscopy negative staining technique, test-plant method, DAS-ELISA tests and RT-PCR method. The following test-plants were inoculated: *Amaranthus caudatus* L., *A. paniculatus* L., *A. retroflexus* L., *Capsicum annuum* L., *Celosia argentea* f. *cristata* L., *Chenopodium amaranticolor* Coste et Reyn., *C. ambrosioides* L., *C. foetidum* Schrad., *C. murale* L., *C. quinoa* Willd., *Datura stramonium* L., *Glycine max* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Nicandra physalodes* L., *Nicotiana affinis* L., *N. benthamiana* L., *N. clevelandi* L., *N. debneyi* L., *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. 'Samsun', *Tetragonia expansa* L., *T. tetragonioides* L., *Vicia faba* L. 'Kupa', *Zinnia elegans* L. Viruses were mechanically inoculated to leaves of young test-plants using carborundum powder as an abrasive. The inoculum was prepared by homogenizing infected leaves with 0.05 M phosphate buffer (pH 7.2) containing 0.4% sodium dithiocarbamate and 0.2% sodium sulphite. Isolates were maintained and propagated in *C. argentea* f. *cristata*, *C. quinoa* and *D. stramonium* plants.

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). ELISA was used as screening test and was performed using "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) immunological kits as described by Clark and Adams (1977). Tests were considered to be positive if the UV absorbance at 405 nm wavelength of investigated sample was equal or greater than 3 times the absorbance of negative (healthy plant) control.

Electron microscopy (EM). Virus particles were observed in negatively stained dip preparations using electron microscope JEOL JEM-100S. Leaves of infected plants were cut in places showing disease symptoms (chlorotic/necrotic lesions or ringspots) as virus concentration is expected to be highest there. Such cut surface then was exposed to the drop of bidistilled water on EM grid and virus particles were precipitated on membrane. The grid then was drained and stained with 2% uranil acetate (Harris, Horne, 1994; Dijkstra, de Jager, 1998).

Purification of RNA. Total RNA extraction was performed with TRIzol reagent ("Invitrogen", USA) according to the protocol as described by Chomczynski and Sacchi (1987) and Zhang et al. (1998).

Reverse transcription-polymerase chain reaction (RT-PCR). Extracted RNA was used for virus detection and identification by RT-PCR. Extracts from healthy plants were used as negative control. Primers for TRSV detection were F: 5'-CTTGCGGCCCAAATCTATAA-3' and R: 5'-ACTTGTGCCCAGGAGAGCTA-3' (Jossey, Babadoost, 2006). Primers for TBRV detection were F: 5'-TCTGGITTTGCYTTRACRGT-3' and R: 5'-CTTRTCACTVCCATCRGTAA-3' (Wei, Clover, 2008). Amplification was performed in "TProfessional Thermocycler" ("Biometra", Germany) using single-step RT-PCR method. Reaction performed in mixtures containing 2 µl of each primer (25 µM), 7.5 µl 10^x Taq buffer, 3.5 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 0.25 µl RevertAid™ reverse transcriptase (200 u μl^{-1}), 0.25 µl Taq polymerase (5 u μl^{-1}) ("Fermentas", Lithuania), 2 µl RNR and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme was used: 42ºC – 30 min, 94ºC – 3 min, 40 cycles [95ºC – 30 s, 55ºC for TRSV or 50ºC for TBRV – 30 s, 72ºC – 1 min] and 72ºC – 5 min. Amplified DNR fragments were analyzed by electrophoresis in 1.5% agarose gel, stained with etidium bromide and visualized in "Bio-DocAnalyze" ("Biometra", Germany) gel documentation system using GeneRuler™ 50 bp DNA ladder marker ("Fermentas", Lithuania).

Results and discussion

Tobacco ringspot nepovirus (TRSV) was isolated from tomato fruits which were hardened with rough surface and exhibiting discoloration both on surface and inside the fruit (Fig. 1 A). Isolate was inoculated to more than 20 different test-plant species (Table).

Results of inoculation were as follows: *Nicotiana glutinosa*, *N. rustica*, *Vicia faba* 'Kupa', *Celosia argentea* f. *cristata*, *Tetragonia expansa*, *Chenopodium ambrosioides*, *Amaranthus caudatus*, *Gomphrena globosa* exhibited necrotic local lesions with bright centre and brown ring around it (Fig. 1 B, C). *Chenopodium amaranticolor*, *C. foetidum*, *C. murale* and *C. quinoa* exhibited yellow local lesions. *Datura stramonium* at first exhibited chlorotic lesions which later became bigger and merged together. Leaves then exhibited mosaic and deformation. Later the plants showed systemic tip necrosis and whole plants withered (Fig. 1 D).

Lycopersicon esculentum showed systemic infection, leaf veins became brown, leaves had yellowish mottle and downward leaf rolling. *Amaranthus paniculatus*, *A. retroflexus*, *Capsicum annuum*, *Glycine max*, *Nicotiana affinis*, *N. benthamiana*, *N. tabacum* 'Samsun', *Zinnia elegans* showed necrotic local lesions, infected plants usually stopped developing and were stunted. *Cucumis sativus*, *Pisum sativum*, *Nicotiana debneyi* and *Nicandra physalodes* showed no signs of virus infection.

Electron microscopy revealed isomeric virus particles of about 30 nm in diameter in preparations made from naturally infected tomato fruits and from various test-plants (*N. glutinosa*, *D. stramonium*, *G. globosa*, *N. rustica*, *C. argentea* f. *cristata*, *C. quinoa*) (Fig. 2).

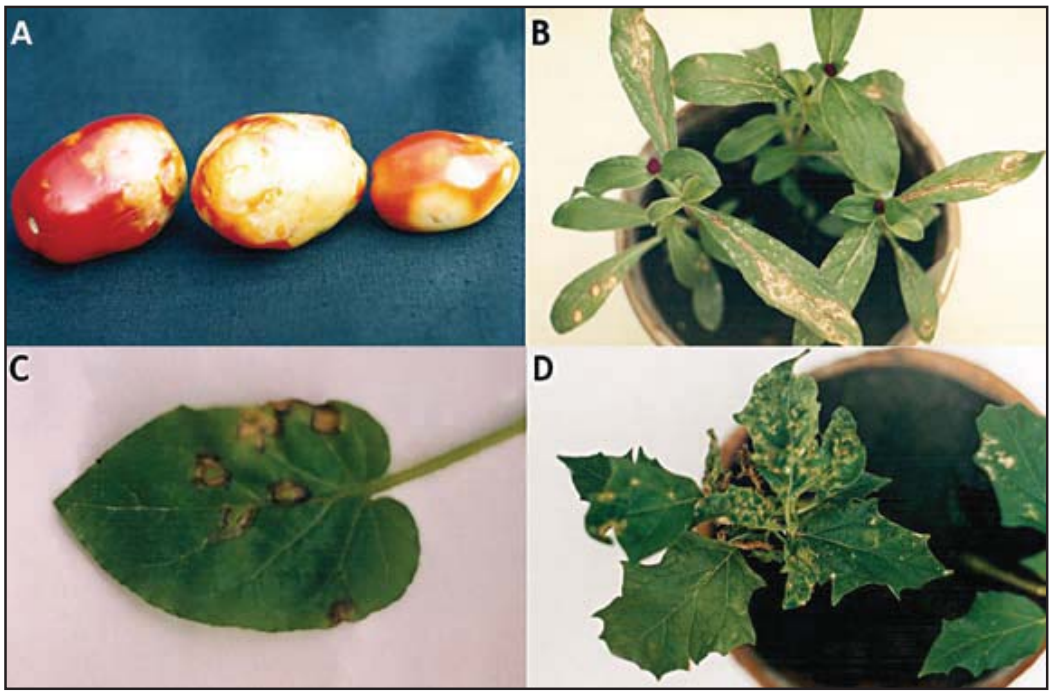

Figure 1. Symptoms of TRSV infection: A – tomato fruits with TRSV, B – *Gomphrena globosa* and C – *Nicotiana glutinosa* showing necrotic ringspots after inoculation with TRSV, D – systemic chlorotic mottle and tip deformation of *Datura stramonium*

Table. Reaction of test-plants to infection of TRSV and TBRV

Test-plant	Symptoms	
	TRSV	TBRV
Amaranthus caudatus L.	NLL, S (St)	–
A. paniculatus L.	NLL, S (St)	–
A. retroflexus L.	NLL, S (TN)	–
Capsicum annuum L.	NLL	–
Celosia argentea f. *cristata* L.	NLL	–
Chenopodium amaranticolor Coste et Reyn.	ChLL	ChLL
C. ambrosioides L.	NLL	–
C. foetidum Schrad.	ChLL	–
C. murale L.	ChLL	ChLL
C. quinoa Willd.	ChLL	ChLL, ChRs
Datura stramonium L.	ChLL, S (ChMo, N)	NRs
Glycine max L.	NLL, S (St)	–
Gomphrena globosa L.	NLL	NLL, VN
Lycopersicon esculentum Mill.	S (VN, ChMo, LR)	–
Nicandra physalodes L.	–	ChLL
Nicotiana affinis L.	NLL	–
N. benthamiana L.	NLL	0
N. clevelandi L.	–	0
N. debneyi L.	S (ChMo)	ChRs, S (ChMo, LD)
N. glutinosa L.	NLL, S (St)	S (ChMo)
N. rustica L.	NLL	NLL, NRs
N. tabacum 'Samsun'	NLL, S (St)	NLL, NRs
Tetragonia expansa L.	NLL	–
T. tetragonioides L.	–	NRs
Vicia faba L. 'Kupa'	NLL	0
Zinnia elegans L.	NLL, S (St)	–

Note. N – necrotic, LL – local lesions, S – systemic, St – stunted, TN – tip necrosis, Ch – chlorotic, Mo – mottle, VN – vein necrosis, LR – leaf roll, Rs – ringspot, LD – leaf deformation, 0 – no symptoms, – not tested.

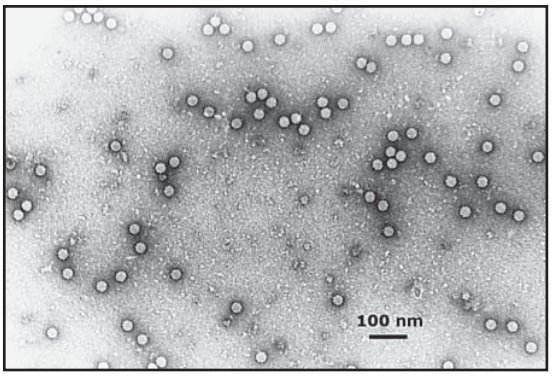

Figure 2. TRSV particles revealed by electron microscopy in extract from leaf of *Datura stramonium*

TRSV was identified in tomato fruits by positive reaction in DAS-ELISA test. Positive reaction in this test was also observed with inoculated test-plants (*G. globosa*, *D. stramonium*, *L. esculentum*, *C. quinoa*).

Total RNA was extracted from naturally infected tomato fruits and infected test-plants (*N. glutinosa* and *D. stramonium*). It was then used to identify TRSV by single-step RT-PCR method using virus-specific primers. The specific PCR product (348 bp) was obtained in all tested samples but not in negative control (healthy *C. quinoa* plant RNA extract) (Fig. 3). In addition to specific product, RNA extracts from samples also yielded smaller PCR products (~290 bp). This may be due to not completely optimized PCR conditions. This investigation was only a first attempt to use single-step RT-PCR for TRSV detection in our laboratory.

Most nepoviruses have a wide host range (both natural and experimental) and can spread through seeds.

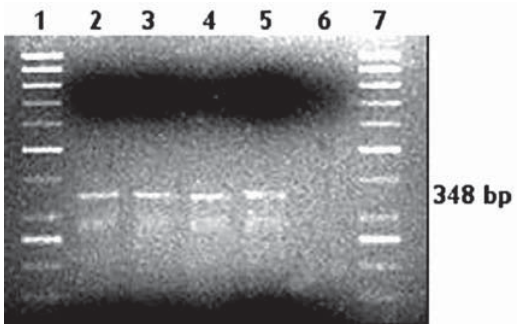

Figure 3. TRSV specific 348 bp PCR product of two virus isolates from tomato fruits and mechanically inoculated test-plants: 1, 7 – marker, 2, 3 – TRSV isolated from different tomato fruits, 4 – *Nicotiana rustica*, 5 – *Datura stramonium*, 6 – negative control

Surprisingly, TRSV isolates appeared to be very aggressive in inoculated test-plants and caused their death. This is incompatible with the ability to spread through seeds. Usually symptoms of nepovirus infection disappear after 2–3 weeks.

Tomato black ring nepovirus (TBRV) was isolated from tomato plant exhibiting chlorotic mottling with a few necrotic lesions and leaf deformation. Isolate was mechanically inoculated to about 15 test-plant species (Table). Results of inoculation were as follows: *Gomphrena globosa* exhibited symptoms similar to TRSV – necrotic local lesions with bright centre and brown ring around it, but also showed vein necrosis. *Chenopodium amaranticolor*, *C. murale* and *C. quinoa* exhibited chlorotic local lesions; on *C. quinoa* these lesions turned into chlorotic ringspots, later, the older leaves withered but the plants grew normally and produced seeds (Fig. 4 A). *Nicotiana glutinosa* exhibited systemic mottling. *N. rustica* and *N. tabacum* 'Samsun' showed necrotic local lesions, some of which later turned into necrotic ringspots. *N. debneyi* showed local chlorotic ringspots and systemic chlorotic mottling with leaf deformation (Fig. 4 B). *Nicandra physalodes* showed yellow local lesions with black outline. *Datura stramonium* and *Tetragonia tetragonioides* exhibited local necrotic ringspots, leaves with local symptoms withered but the plants grew normally (Fig. 4 C, D). *Nicotiana clevelandi*, *N. benthamiana* and *Vicia faba* 'Kupa' showed no signs of virus infection.

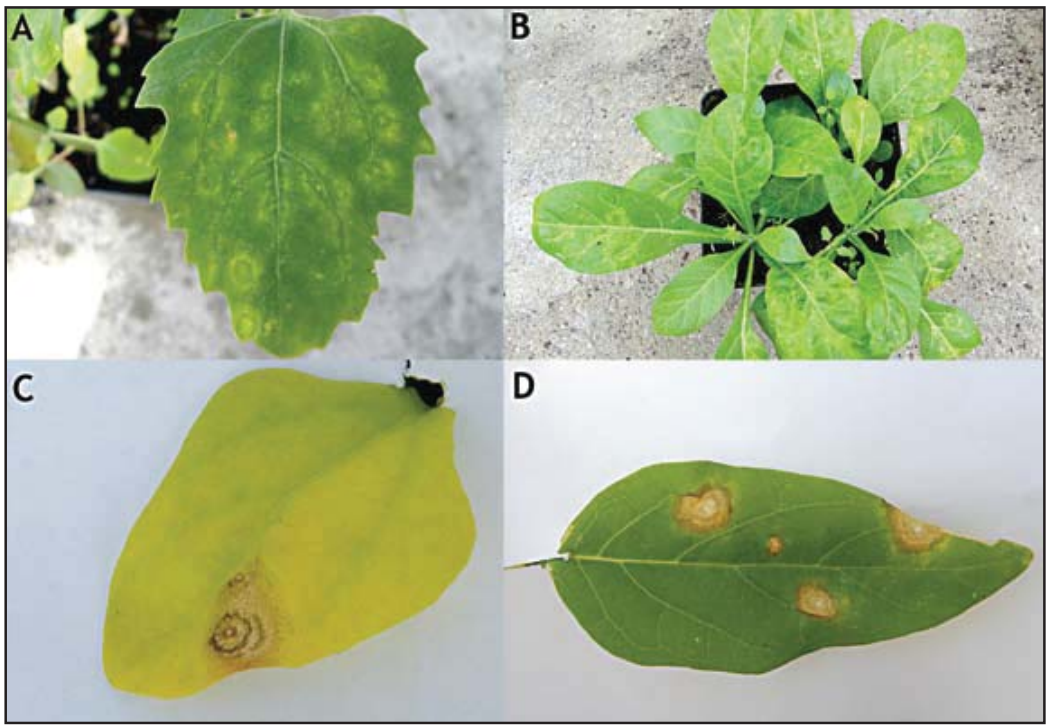

Figure 4. Symptoms of TBRV infection: A – chlorotic ringspots on *Chenopodium quinoa* leaf, B – *Nicotiana debneyi* showing systemic chlorotic ringspots and mottle, C – leaf of *Tetragonia tetragonioides* and D – cotyledon of *Datura stramonium* with necrotic ringspots

Electron microscopy revealed isomeric virus particles of about 30 nm in diameter in preparations made from various test-plants (*D. stramonium*, *N. debneyi*, *N. rustica*, *T. tetragonioides*) (data not shown).

TBRV was identified in naturally infected tomato plant by positive reaction in DAS-ELISA test as well as in some inoculated test-plants (*D. stramonium*, *C. quinoa*, *T. tetragonioides*).

Total RNA was extracted from naturally infected tomato and *D. stramonium*, *C. quinoa*, *T. tetragonioides*, *N. debneyi* test-plants. It was then used to identify TBRV by single-step RT-PCR method using primers specific to nepoviruses belonging to subgroup B. The specific PCR product (221 bp) was obtained in all tested samples but not in negative control (healthy *C. quinoa* plant RNA extract) (Fig. 5). RNA extract from *N. debneyi* also yielded

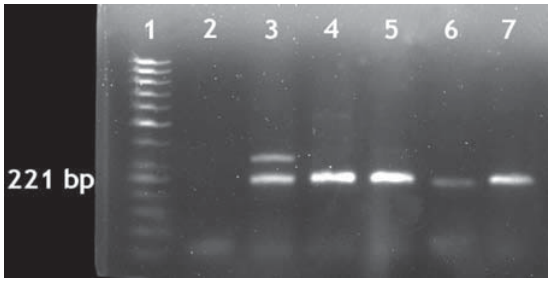

Figure 5. TBRV-specific 221 bp PCR product from naturally infected tomato and mechanically inoculated test-plants: 1 – marker, 2 – negative control, 3 – *Nicotiana debneyi*, 4 – *Datura stramonium*, 5 – *Chenopodium quinoa*, 6 – *Tetragonia tetragonioides*, 7 – naturally infected tomato

additional bigger PCR product which was absent in other isolates. Hence, this product may have been amplified from plant RNA and not from viral.

Both nepoviruses could be harmful, because their vectors are present in Lithuania and wild weeds could provide means to spread to economically important plants. It is difficult to estimate damage done by TBRV, because virus infection often can be symptomless and remain undetected.

Conclusion

Two viruses found in tomato plants were identified as a *Tobacco ringspot nepovirus* (TRSV) and *Tomato black ring nepovirus* (TBRV), applying serological and molecular methods. Both TRSV and TBRV have been found naturally infecting tomatoes in our country for the first time, though TRSV has already been found widespread in Lithuania, infecting a wide range of ornamental plants.

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Nepovirusų identifikacija valgomojo pomidoro (*Lycopersicon esculentum* Mill.) augaluose ir vaisiuose

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Santrauka

Lietuvoje anksčiau buvo nustatyta keletas nepovirusų (vaistučio mozaikos virusas, tabako žiediškosios dėmėtligės virusas ir pomidorų žiediškosios dėmėtligės virusas), infekuojančių dekoratyvinius augalus. Straipsnyje pateikti duomenys apie dviejų nepovirusų – tabako žiediškosios dėmėtligės viruso (TRSV) ir pomidorų juodojo žiediškumo viruso (TBRV) – identifikaciją pomidoruose. Virusinės infekcijos simptomų turintys pomidorai tirti imunofermentinės analizės (ELISA) metodu. Augalų, rodžiusių teigiamą reakciją taikant ELISA testus, ekstraktai buvo mechaniškai inokuliuoti į daugiau nei dvidešimt skirtingų augalų indikatorių rūšių. Augalai, priklausantys *Amaranthus*, *Nicotiana*, *Chenopodium*, *Datura*, *Gomphrena* ir *Tetragonia* gentims, reagavo į virusus, ant jų lapų atsirado vietinės pažaidos, taip pat vystėsi sisteminės infekcijos požymiai. Buvo atlikta įvairių mechaniškai užkrėstų augalų ekstraktų elektroninė mikroskopija (EM). Preparatuose aptiktos nepovirusams būdingos ~30 nm sferinių virusų dalelės. TRSV ir TBRV nepovirusų infekcija patvirtinta ir taikant atvirkštinės transkripcijos polimerazės ciklinės reakcijos (AT-PCR) metodą, naudojant šiems virusams specifinius PCR pradmenis. Taigi, TRSV bei TBRV virusai buvo identifikuoti pomidoruose ir mechaniškai užkrėstuose augaluose ELISA, EM ir AT-PCR metodais.

Reikšminiai žodžiai: *Tobacco ringspot nepovirus*, *Tomato black ring nepovirus*, AT-PCR, identifikacija.