ISSN 1392-3196
Žemdirbystė=Agriculture, vol. 99, No. 1 (2012), p. 85–92
UDK 634.22:633.63:632.3

Detection and molecular identification of alien viruses of plums, sugar beets and tomatoes

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Abstract

Alien virus species infecting stone fruit and vegetable crops in Lithuania are considered to be: *Plum pox virus* (PPV) affecting plum (*Prunus domestica* L.), *Beet necrotic yellow vein virus* (BNYVV) affecting sugar beet (*Beta vulgaris* var. *saccharifera* L.) and *Pepino mosaic virus* (PepMV) affecting tomatoe (*Lycopersicon esculentum* Mill.). The viruses were isolated from natural infection sources and mechanically transmitted to susceptible test-plants. Identity of PPV in symptomatic plum tree leaves and test-plants (*Chenopodium foetidum*, *Pisum sativum*), BNYVV in sugar beet roots and test-plants (*Chenopodium quinoa*, *C. amaranticolor*, *Tetragonia expansa*) and PepMV in tomato fruits and *Nicotiana* test-plant species was confirmed by immuno enzyme DAS-ELISA test, electron microscopy, and molecular RT-PCR procedure. RT-PCR analysis confirmed all PPV isolates to be of PPV-D strain. PPV appeared to have limited distribution in a few administrative regions of central and northern part of Lithuania. BNYVV was detected in four administrative regions and it seems that B type of BNYVV is predominant in Lithuania. PepMV at first was detected and identified in imported commercial tomato fruits. Later on this virus was detected in commercial tomato greenhouses in Lithuania. Isolates from tomato fruits were identified as PepMV on the basis of symptom expression on the fruits and test-plants, morphology of virions, results of DAS-ELISA tests and RT-PCR analysis.

Key words: *Plum pox virus*, *Prunus domestica*, *Beet necrotic yellow vein virus*, *Beta vulgaris* var. *saccharifera*, *Pepino mosaic virus*, L*ycopersicon esculentum*, identification, RT-PCR.

Introduction

Emerging infectious diseases are caused by pathogens that have increased incidence, geographical or host range, have changed pathogenesis, have newly evolved or have been discovered or newly recognized (Anderson et al., 2004). Alien to Lithuania viruses could be considered those that previously emerged in different geographical regions and their host range and prevalence have been increasing. Increased international trade of seed, seedlings, cuttings and fruit enhances the risk of introducing new viruses and their vectors. Moreover, changing climate conditions can contribute to a more successful spread of alien viruses. *Plum pox virus* (PPV) affecting plums (*Prunus domestica* L.), *Beet necrotic yellow vein virus* (BNYVV) affecting sugar beets (*Beta vulgaris* var. *saccharifera* L.) and *Pepino mosaic virus* (PepMV) affecting tomatoes (*Lycopersicon esculentum* Mill.) are considered to be alien viruses infecting stone fruit and vegetable crops in Lithuania. All three viruses emerged in warmer climate conditions, gradually increased their incidence, host range and pathogenesis. The main characteristics of the alien viruses in Lithuania are presented in Table 1.

Plum pox virus. PPV causes a devastating stone fruit disease known as "Sharka" and can provoke a serious economic impact. Stone-fruits infected by PPV include wild and cultivated *Prunus* spp.: plum, peach, apricot, almond, nectarine, sweet cherry and sour cherry. PPV or 'Sharka' disease originally described in Bulgaria on *Prunus domestica* L. has gradually spread throughout European borders (Šutič et al., 1999). The disease also affects some wild *Prunus* species, especially blackthorn (*Prunus spinosa* L.) which has been a natural source of infection in many countries. PPV is now reported in most European countries, in parts of Asia and Northern Africa, and in South America. Plum pox is the most serious disease of plums, apricots and peaches in Europe and is considered as a quarantine pathogen in all continents (Šutič et al., 1999). Plum pox symptoms can vary greatly, making the disease difficult to recognize. Visual symptoms are not always a reliable indicator of the disease. Disease symptoms are most noticeable during early summer period. Then leaves can show chlorotic spots, bands, or rings, vein clearing and leaf deformation. Infected fruits can be deformed, show chlorotic spots or rings on the surface,

contain internal browning of the flesh and pale rings or spots on the stones. PPV belongs to the most numerous filamentous mechanically and by aphid transmissible plant virus group – *Potyviruses*. PPV virions are filamentous particles ca. 660–770 × 12.5–20 nm. It is known that PPV has four major strain groups – Dideron (D), Marcus (M), El-Amar (EA) and Cherry (C). European PPV isolates according to serological, biological and other properties can be divided into two major subgroups: PPV-M and PPV-D. In nature, these isolates can show significant differences in their epidemiological properties and host range (Myrta et al., 1996). Severity of crop losses often depends on the strain of the virus, so it is important to identify the most common strain of PPV in our investigation region. Strains of PPV-D group are less aggressive than PPV-M and have limited host range.

Table 1. Main features of the alien viruses affecting stone fruits, sugar beet and tomato in Lithuania

No.	Main features	Plum pox (*Plum pox virus*)	Sugar beet rhizomania (*Beet necrotic yellow vein virus*)	Pepino mosaic (*Pepino mosaic virus*)
1.	Taxonomic status	*Potyviridae* family *Potyvirus* genus	*Benyvirus* genus	*Flexiviridae* family *Potexvirus* genus
2.	Genome	ss RNA, positive-sense	ss RNA, positive-sense	ss RNA, positive-sense
3.	Virion particle morphology	filamentous, ~750 nm	different length rod-like	filamentous ~500 nm
4.	Means of transmission	aphids, nonpersistently	Zoosp. parasitic protozoan *Polymyxa betae*	bumblebee, fungi, mechanical
5.	Main test-plants	*Chenopodium foetidum*, *Pisum sativum*	*Chenopodium, Tetragonia*	*Datura*, *Nicandra*, *Nicotiana*
6.	First detected in Europe / Lithuania	1917 / 1996	1956 / 2004	1999 / 2009
7.	Region of origin	Balkans	Mediterranean (Italy)	South America (Peru)
8.	Symptoms on economic crops	plum leaf mottle, fruit deformation	sugar beet root excessive proliferation (rhizomania)	Tomato fruit mottling, marbling

First evidence of the presence of PPV in Lithuania was obtained at the Lithuanian Institute of Horticulture by Dr. Jūratė Stankienė. ELISA tests indicated the presence of the virus in some plum trees from Kaunas region in about 1995. The Lithuanian isolate appeared to be attributed to D-group of strains, as was identified using monoclonal antibodies in ELISA tests conducted by Dr. Donato Boscia (de Bari, Italy) (Staniulis et al., 1998). More extensive surveys on spread of PPV using ELISA test were carried out at Phytosanitary Research Laboratory of the Lithuanian State Plant Protection Service. The spread of PPV in plum trees in Kaunas region was confirmed in 1997 and forthcoming years. The virus-positive samples detected in 2005 were from three previously defined locations and in 2006 from two. In recent years, PPV has been detected only in two locations. The status of PPV in Lithuania now can be described as: limited presence only in some areas. This study presents results of molecular identification of PPV isolates detected during the past few years.

Rhizomania of sugar beet. Rhizomania of sugar beet (*Beta vulgaris* var. *saccharifera* L.) caused by *Beet necrotic yellow vein virus* (BNYVV), the type member of the genus *Benyvirus*, is one of the most destructive diseases of sugar beet (Tamada, Baba, 1973). Losses of sugar beet root yield can reach 50–60%. It is transmitted by protozoan vector (*Polymyxa betae* Keskin) which survives in infested soil for many years in clusters of thick-walled resting spores, termed sporosori. Therefore, the disease cannot be controlled by crop-rotation or by having a beet-free period (Rush, 2003). The main symptoms of rhizomania are root bearding (known as "root madness"), stunting, chlorosis of leaves, yellow veining and necrosis of leaf veins. The disease is spread by movement of soil, primarily on machinery, sugar beet and other crops roots, and in compost and soil (Rush, 2003). Rhizomania originally described in Italy in 1952 (Canova, 1959) has spread to many other sugar beet growing countries in Europe, Asia and North America (Ratti et al., 2005) and is likely to continue its spread across the world (McGrann et al., 2009). BNYVV has a multipartite single-stranded RNA genome with all natural isolates containing four RNA species, although some isolates have a fifth RNA (Tamada et al., 1989). The larger RNA-1 and RNA-2 are always required for infection; encode proteins involved in viral replication, the encapsidation process, transmission by *P. betae* and cell-to-cell movement. RNA-3 and RNA-5 encode pathogenicity factors responsible for rhizomania symptoms. RNA-4 encodes a 31-kDa protein enhancing vector transmission, symptom severity and RNA silencing suppression in roots. RNA-5-containing isolates are restricted to Asia and some parts of Europe and these isolates tend to be more aggressive (McGrann et al., 2009). Rhizomania disease agent in Europe indicates its tendency of gradual spreading from Southern to Northern Europe, reaching northern sugar beet growing regions. Recently the first detection of BNYVV was recognized in Sweden (Lennefors et al., 2000) and Denmark (Nielsen et al., 2001). Molecular studies based on RT-PCR and restriction fragment length polymorphism (RFLP) analysis have revealed that two major strain groups of the virus (A and B types) containing only four RNA species are prevailing in Europe. The A type is widespread in Southern Europe, the type B predominates in Germany and France, whereas in the United Kingdom both the A and the B types and also mixed infections were found. Type P of BNYVV contains RNA 5, has limited distribution in France and UK and is known to be responsible for severe rhizomania symptoms (Ratti et al., 2005).

Surveys of sugar beet crops for the presence of BNYVV in Lithuania have been regularly carried out since

1998. Sugar beet crop covers more than 20 000 ha. The first outbreak of the disease was reported in 2004 (Jackeviciene et al., 2005) and has since been discovered in other two administrative regions in central Lithuania (Žižytė et al., 2006). In recent years BNYVV has been detected in new region (Kėdainiai).

Pepino mosaic virus. PepMV is an important new pathogen infecting tomato (*Lycopersicon esculentum* Mill.) crops in Europe (Van der Vlugt et al., 2002). Originally PepMV was isolated from infected pepino (*Solanum muricatum* Ait.) plants in the regions of Peru (Jones et al., 1980). Recently surveys carried out in Central and Southern Peru showed that the virus was naturally present in wild *Lycopersicon* species as well as in cultured tomato and pepino (Soler et al., 2002). In European tomato crops PepMV first appeared in 1999. Since then PepMV occurrence has been confirmed in many countries of EC and elsewhere (Canada, Morocco, Ukraine and USA) (Spence, 2001). The virus was detected also in Germany, Tunisia, Sweden, Finland, North America (Hanssen, Thomma, 2010). The virus is highly contagious and rapidly spreads by mechanical means. Its experimental host range is narrow, infecting 30 of 32 solanaceous species, including tomato and potato. Disease symptoms vary greatly depending on strain of the pathogen, tomato variety and season of year. Most often leaf distortion, yellow mottling or mosaic, roughness symptoms are displayed. On fruit surface yellow mottled discoloration and colourless stretches penetrating into deeper tissue could be noticed. It is unlikely that PepMV has true seed transmission. Possibility exists that the virus may be transmitted on contaminated debris from infected tomato on seed when infectious sap has dried. This can be one of the possible explanations of ways for virus long-distance spread. Investigation of possible reservoir hosts among wild plants around greenhouses affected by PepMV from different regions of Spain using DAS-ELISA revealed that *Amaranthus* sp., *Malva parviflora*, *Nicotiana glauca* Graham., *Solanum nigrum* L. and *Sonchus oleraceus* L. contained natural infection. The presence of PepMV in symptomless plants of these weed species was confirmed by electron microscopy, mechanical inoculation of tomato and by RT-PCR (Jorda et al., 2000). The new disease has become a serious problem for tomato production in Europe and its agent is becoming one of the most important tomato pathogens in Europe.

In the beginning of 2002, a new tomato disease in Lithuania was observed in imported tomato fruits. In commercial lots imported from Spain, some fruits have been found showing irregular discoloration and surface roughness. Initial diagnostic tests suggested the presence of PepMV. In preparations from such fruit extracts flexuous virus particles about 500 nm long characteristic of potexvirus were observed by transmission electron microscopy. Analysis of samples from the tomato fruits by DAS-ELISA revealed presence of PepMV recently detected and identified in tomato crops of many European countries. Isolates of the virus obtained from imported tomato fruits in Lithuania have been characterized and identified by test-plant reaction, morphology of virus particles and data of DAS-ELISA test (Zitikaitė et al., 2003). This paper presents new data on specific detection, and molecular identification of PepMV isolates from tomato grown in Lithuania.

Materials and methods

Virus source, electron microscopy and serological methods. As a first stage for identification of all three viruses plant tissue samples from fields were assayed in the Plant Virus Laboratory of the Institute of Botany in 2010–2011 by DAS-ELISA analysis with polyclonal antibodies using immunological kits of "Bioreba" (Switzerland), "Loewe" or DSMZ (Germany), according to described procedures (Clark, Adams, 1977) and electron microscopy (EM) (Brlansky, Derrick, 1979). Intensity of ELISA reactions in microplate wells was estimated photometrically at 405 nm wave length using "Multiskan Microplate Reader" (Finland). The ELISA test was considered to be positive if absorbance of the investigated sample was equal to or greater than 3 times the absorbance of the negative control. Electron microscopic investigation of negatively stained with 2% uranyl acetate dip preparations were performed with "Jeol JEM-100S" transmission electron microscope at instrumental magnification of 25000^{x}. In the cases of low virus concentration immunosorbent electron microscopy (ISEM) was used (Dijkstra, de Jager, 1998). Plant tissue samples with ELISA positive results were used for PCR procedures and mechanical inoculation set of test-plants using buffer solutions containing stabilizing agents (1.5% nicotine, 0.25% Na DIECA and 0.2% Na_2SO_3). Total RNA extraction was performed either by the CTAB (hexadecyltrimetylamoniumbromid) based method (Chang et al., 1993) or TRIzol reagent ("Invitrogen", USA) according to the protocol as described by Chomczynski and Sacchi (1987). The resulting RNA was dissolved in 30–40 µl diethyl pyrocarbonated (DEPC)-treated water.

PPV detection by RT-PCR. RT-PCR with primers for general PPV detection (Wetzel et al., 1991) was performed in mixtures containing 2 µl of each primer (25 µM), 7.5 µl 10 × *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 of extracted RNR and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme was used: 42ºC for 30 min, 94ºC for 3 min, 40 cycles of 94ºC for 30 s, 54ºC for 30 s, 72ºC for 30 s followed by 72ºC for 5 min.

PPV subgroup was identified by integrated RT-PCR/nested PCR with primers reported by Szemes et al. (2001). Reaction was performed in mixtures containing 2 µl of M2 primer (25 µM), 1 µl of M3 primer (25 µM), 1 µl of M3 primer (25 µM), 7.5 µl 10 × *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 of extracted RNR and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme was used: 42ºC for 30 min, 94ºC for 3 min, 40 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min followed by 72ºC for 5 min. The amplified products then were used in the second step of nested PCR using subgroup-specific primer pairs. Nested PCR was performed in mixtures containing 2 µl of each subgroup-specific primer (25 µM), 5 µl 10 × *Taq* buffer, 2 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 0.25 µl *Taq* polymerase (5 u μl^{-1}) ("Fermentas", Lithuania), 1 µl of previously amplified DNA and DEPC H_2O up to a total volume of 50 µl. The following cycling scheme was used: 94ºC for 3 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min followed by 72ºC for 5 min.

Identification of BNYVV. RT-PCR was carried out using specific primers, which amplify coat protein (CP) gene located on RNA-2 (Schirmer et al., 2005). First strand synthesis of BNYVV cDNA was carried out using 2 µl of reverse primer (20 µM) with 3 µl of total RNA and 6 µl of DEPC-treated water. The mixture was incubated at 70°C for 5 min and chilled on ice prior to the addition of 1.5 µl of DEPC-treated water, 2 µl of dNTPs (10 mM), 4 µl of RT 5 × buffer, 0.5 µl Ribonuclease inhibitor (40 U μl^{-1}) and 1 µl of RevertAid™ reverse transcriptase (200 U μl^{-1}) ("Fermentas", Lithuania). The reverse transcription reaction was carried out at 42°C for 60 min. PCR amplification was carried out using a special PCR mix (20 µM specific forward and reverse primers, 10 × *Taq* reaction buffer, 25 mM $MgCl_2$, 10 mM dNTPs and 5 u μl^{-1} *Taq* DNA polymerase ("Fermentas", Lithuania). Thermocycling temperature for BNYVV CP gene amplification was 94°C for 3 min, 35 cycles of 94°C for 1 min, 61°C for 1 min and 74°C for 1 min, followed by 72°C for 10 min.

BNYVV type determination was done by multiplex RT-PCR (mRT-PCR). For mRT-PCR a two-stage PCR was used: in the first stage RT-PCR was carried out using specific primers for TGB gene amplification. In the second stage, nPCR was carried out using previously amplified TGB fragment and A/B type-specific primers (Ratti et al., 2005).

Detection of PepMV by RT-PCR. RT-PCR for PepMV identification was performed with primers reported by Mumford and Metcalfe (2001). The first strand cDNA synthesis was carried out at 37ºC for 60 min and 70ºC for 10 min using PCR mix of 20 µM specific reverse primer, RT 5 × buffer, 10 mM dNTPs, ribonuclease inhibitor (40 U μl^{-1}) and RevertAid™ reverse transcriptase (200 U μl^{-1}) ("Fermentas", Lithuania). DNA amplification was performed using reaction mixture containing dNTP, both primers and recombinant *Taq* DNA polymerase ("Fermentas", Lithuania). Thermocycling temperature for PCR was 94°C for 4 min, 40 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min followed by 72°C for 10 min.

All PCR amplifications were performed in "TProfessional Thermocycler" ("Biometra", Germany). Amplified DNR fragments were analyzed by electrophoresis in 1.5–2% agarose gel, stained with ethidium bromide and visualized in "BioDocAnalyze" ("Biometra", Germany) gel documentation system using GeneRuler™ 50 bp DNA ladder marker ("Fermentas", Lithuania).

Results

Plum pox virus. Nearly one hundred samples of plum and peach tree leaves with characteristic mottle symptoms were collected from various plum nurseries and orchards of the main plum growing locations. The samples were assayed for PPV by ELISA test, immunosorbent electron microscopy (ISEM) and the RT-PCR. DAS-ELISA was conducted at the Institute of Botany and State Plant Protection Service. All PP positive plum and peach leaf samples had symptoms rather characteristic of PPV – slight mosaic or ring mottling (Fig. 1 A and B).

Figure 1. PPV symptoms on plum (*Prunus domestica* L.) leaves (A) and peach (*Prunus persica* L.) leaves (B)

From the 92 collected samples PPV was identified in three isolates (in two different plum trees and one peach) by positive reaction in DAS-ELISA tests. DAS-ELISA positive samples were subjected to EM investigation and particles typical of potyvirus filamentous virus were detected (Fig. 2).

Several isolates from the symptomatic and ELISA positive plum leaf were mechanically transferred to herbaceous plant species *Chenopodium foetidum* Schrad. and *Pisum sativum* L. which reacted by expressing local lesions and systemic infection, respectively. Total RNA extracted from infected plant leaves was used to confirm PPV identification by single-step RT-PCR using general PPV detection primers P1 and P2 (Wetzel et al., 1991).

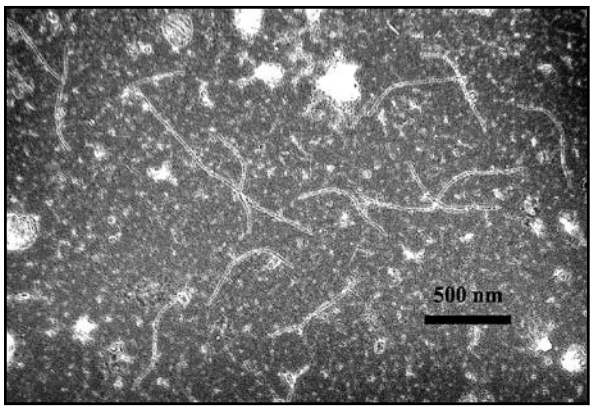

Figure 2. Filamentous PPV virus particles in symptomatic plum tissue extract (ISEM preparation)

The specific PCR product (243 bp) was obtained in positive control and all tested samples but not in negative control (healthy *Prunus* plant RNA extract) (Fig. 3).

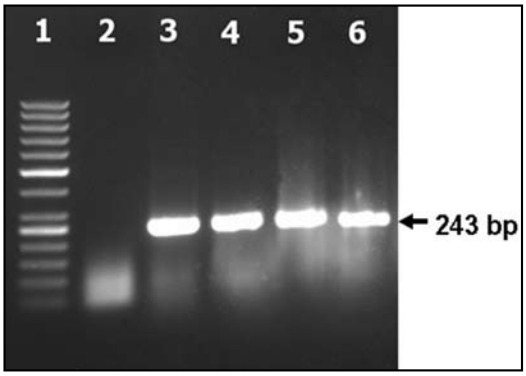

Figure 3. PPV specific 243 bp PCR product from infected plants: 1 – GeneRuler™ 50 bp DNA ladder, 2 – negative control, 3 – isolate from plum No. 1, 4 – isolate from plum No. 2, 5 – isolate from peach, 6 – positive control

After confirming the presence of virus, strain specificity of PPV was determined by integrated RT-PCR/nested PCR. At first, single-step RT-PCR was carried out using mixture of M3, M4 and M2 primers reported by Szemes et al. (2001). Obtained PCR products then were used in second step of nested PCR using three different strain-specific primer pairs. All three tested samples yielded PCR product (159 bp) specific to PPV-D strain (Fig. 4). Since no products were amplified with PPV-M and PPV-E specific primer pairs, it means that all three virus isolates belong to PPV-D strain. PPV-D strain was also determined in previously found PPV isolates in Lithuania (Norkus et al., 2008). In Lithuania, natural sources of PPV infection are not known. PPV-D strain group isolates cause milder symptoms, thus virus infection can easily be overlooked in grafting or propagating material and virus introduced. It is quite possible that in Lithuania natural sources of PPV infection are not present.

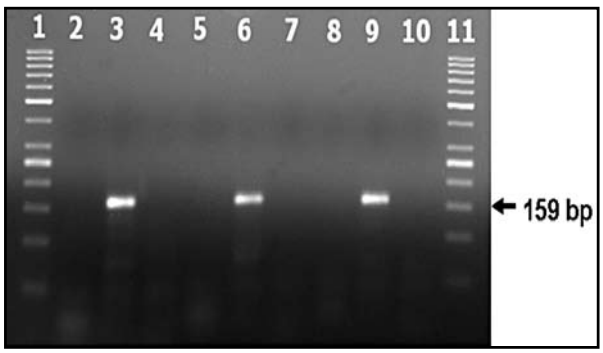

Figure 4. PPV-D specific 159 bp PCR product: 1, 11 – GeneRuler™ 50 bp DNA ladder, 2, 3, 4 – plum No. 1 isolate reactions with M, D and E specific primer pairs, respectively, 5, 6, 7 – plum No. 2 isolate reacts with M, D and E specific primer pairs, respectively, 8, 9, 10 – peach isolate reacts with M, D and E specific primer pairs, respectively

Beet necrotic yellow vein virus. Rhizomania virus of sugar beet was identified in three administrative districts (Kaunas, Panevėžys and Šakiai) of Lithuania (Jackeviciene et al., 2005; Žižytė et al., 2006) and infection level appeared to be low. In 2009, BNYVV was confirmed just in one location of Kaunas region. In recent years (2010 and 2011), sugar beet root samples were collected from various sugar beet growing regions of Lithuania (Kaunas, Kėdainiai, Marijampolė, Panevėžys, Šakiai, Vilkaviškis districts). Collected sugar beet roots with rhizomania symptoms were analyzed using DAS-ELISA test, immuno lateral flow test and by ISEM with trapped antibodies against BNYVV. Out of the 224 sugar beet samples tested BNYVV was detected just in two root samples collected in Panevėžys region and one in Kėdainiai region. ISEM revealed numerous trapped straight very short and longer rod-shaped virus particles about 20 nm in diameter characteristic of rhizomania agent (Fig. 5).

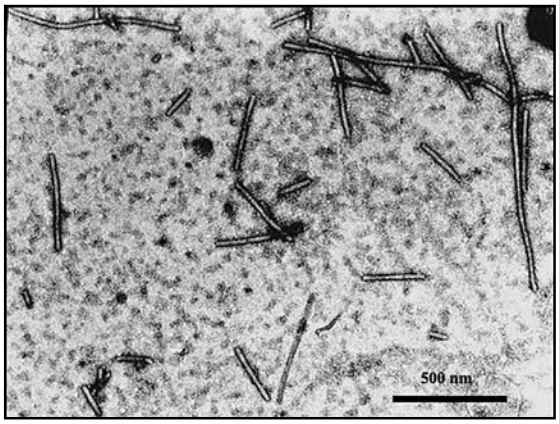

Figure 5. Rod shaped virus particles in BNYVV infected plant tissue

Mechanically inoculated test-plants (*Chenopodium amaranticolor*, *C. quinoa*, *Tetragonia expansa*) developed local chlorotic lesions. Tissue extracts of infected test-plants with local lesions were used for BNYVV propagation, RT-PCR analysis and BNYVV type determination. BNYVV identification was also confirmed by RT-PCR using primers (Schirmer et al., 2005), which amplify CP gene located on RNA-2. Specific product of 567 bp length was obtained in these investigated samples (Fig. 6).

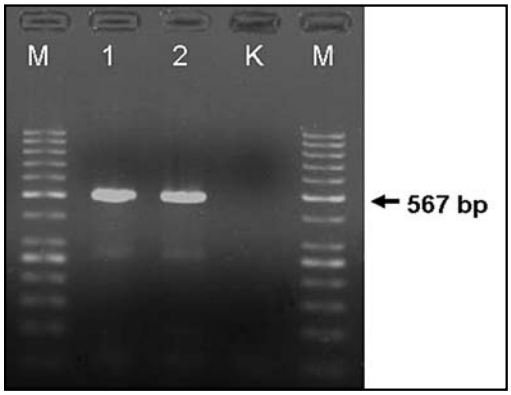

Figure 6. RT-PCR detection of BNYVV coat protein gene: M – GeneRuler 50 bp DNA ladder, 1 – isolate from Kėdainiai, 2 – isolate from Panevėžys, K – negative control

The types of detected BNYVV isolates were determined using multiple RT-PCR (mRT-PCR) which was performed in two stages. The first RT-PCR stage was carried out using specific primers for triple gene block (TGB) fragment. In the second stage nested PCR was carried out using amplified TGB gene fragment, and two pairs of specific primers for BNYVV A and B type. All tested samples yielded PCR product specific for B type (178 bp). No amplification product was observed in healthy *C. quinoa* plant (Fig. 7).

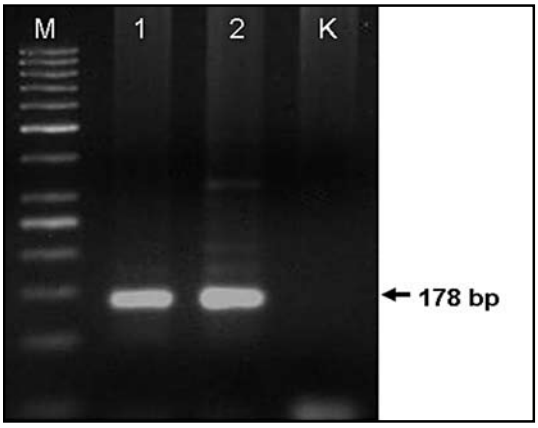

Figure 7. Type determination of Lithuanian BNYVV isolates by mRT-PCR: A/B type products: M – GeneRuler 50 bp DNA ladder, 1 – isolate from Kėdainiai, 2 – isolate from Panevėžys, K – negative control

Pepino mosaic virus. In 2011, 85 samples of tomato fruits and leaves with symptoms of possible virus infection were collected from greenhouses of 6 administrative regions in Lithuania and 20 samples of fruits from supermarkets. Symptoms on tomato leaves suspected to have been caused by the virus infection were: light green lamina, vein clearing, mottling, chlorotic spots, deformation, rugose. Tomato fruits having uneven red discoloration, rough surface with yellowish stretch, or dim spots penetrating into deeper tissues layers giving marble tint were collected from greenhouses and supermarkets supplied by local growers. Mild symptoms of PepMV on DAS-ELISA positive tomato fruits are represented in Figure 8.

Figure 8. Symptoms of PepMV infection on tomato fruits

Tomato fruit samples collected from greenhouses of Kaunas, Kaišiadorys and Panevėžys regions decisively demonstrated presence of PepMV. In 17 cases DAS-ELISA positive fruit tissue samples, imported from Spain, the Netherlands and Poland, were applied for mechanical inoculation of set of 14 test-plant species in order to detect possible symptom expression or host range differences. Most susceptible plant species appeared to be *Lycopersicon esculentum*, *Datura stramonium*, *Nicotiana benthamiana* and *N. occidentalis*. Usually, only bright irregular chlorotic spots appeared on leaves of experimentally inoculated tomato. Different degree of susceptibility could be expected among tomato cultivars. EM investigation of infected plant fruit tissue indicated the presence of potexvirus-like virions. In tissue of inoculated *Nicotiana glutinosa*, *N. rustica*, *N. debneyi* and *N. tabacum* plants, the presence of PepMV was not confirmed. However, PepMV isolates from Spain infected *N. debneyi* and *N. rustica* plants as well. Experimental infection of *Helleborus niger* and *Tetragonia expansa* should be noticed, though the latter is not a member of *Solanaceae* Juss. family. Thus, according to our data *T. expansa* could be considered as a new diagnostic plant for PepMV. No significant symptom expression differences among Lithuanian isolates were detected.

EM or ISEM investigation was carried out as an additional means for confirmation of PepMV infection in tomatoes of local production. Filamentous rod-like particles were usually detected in ELISA-positive fruit tissue and test-plant leaf samples. The particle morphology was characteristic of genus potexvirus, about 500 nm in length (Fig. 9).

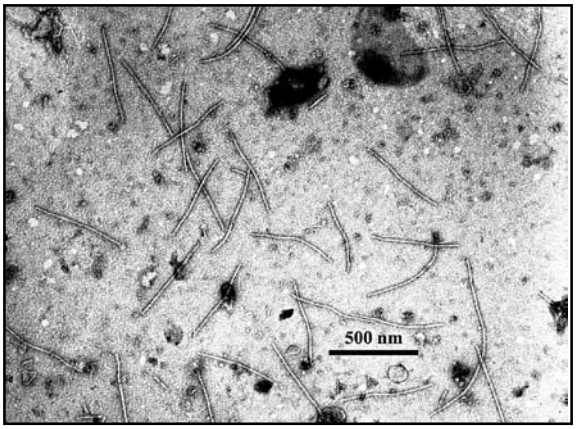

Figure 9. Potexvirus characteristic particles in tomato fruit tissue extract prepared by ISEM procedure

PCR procedures for PepMV identification were repeated several times employing different protocols and using different primers. Most suitable appeared to be Mumford and Metcalfe (2001) reported primers, amplifying 844 bp DNA fragment. Tomato fruit samples with characteristic symptoms of PepMV infection imported from Spain, and Poland (data not shown), the Netherlands in RT-PCR procedures indicated presence of PepMV. This virus was also detected in tomato production supplied from greenhouses of domestic growers and tomato samples collected directly from the greenhouses of growers (isolates KK and NV from Elektrėnai and Kaunas region, respectively). Test-plant samples infected by

PepMV in RT-PCR procedures amplified product of 844 bp (Figs 10 and 11).

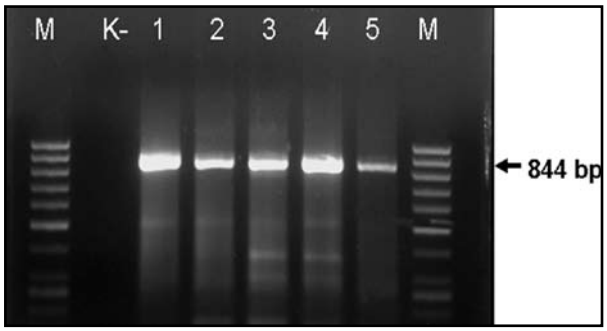

Figure 10. RT-PCR products from PepMV inoculated test-plants: M – GeneRuler™ 50 bp DNA ladder, K- – negative control, 1 – tomato sample from Holland (No. 37), 2 – *Datura stramonium* (inoculated from isolate KK), 3 – *Lycopersicon esculentum* test-plant inoculated from isolate KK, 4 – *Tetragonia expansa* test-plant inoculated from isolate KK, 5 – *Nicotiana occidentalis* test-plant inoculated from Holland tomato

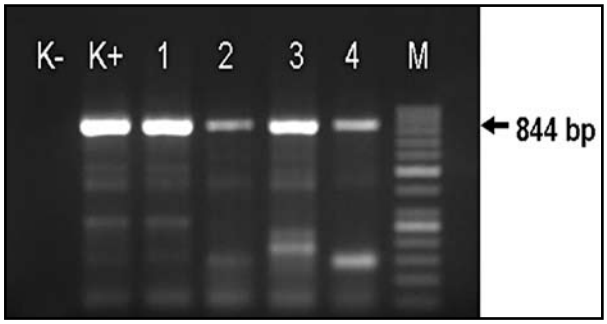

Figure 11. RT-PCR of tomato samples collected from greenhouse in Kaunas region (NV). PCR amplification product – 844 bp. K− – negative control, K+ – positive control, 1–4 – tomato samples from greenhouse NV in Kaunas region, M – GeneRuler™ 50 bp DNA ladder

Conclusions

1. The presence of *Plum pox virus* in plum and peach tree samples from Lithuania was confirmed by DAS-ELISA, ISEM and RT-PCR methods. In all samples analyzed, only PPV-D strain of the virus was proved.

2. *Beet necrotic yellow vein virus* appeared to have limited distribution and was detected only in three administrative regions. All BNYVV isolates should be attributed to B strain group.

3. *Pepino mosaic virus* was detected and identified only in greenhouse grown tomato.

4. No data were collected about the tendencies of distribution or prevalence in Lithuania-identified alien viruses.

Acknowledgements

The present study was supported by the grant from Research Council of Lithuania No. LEK 16/2010, project “Fitovirusai”.

Received 15 11 2011
Accepted 16 12 2011

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ISSN 1392-3196
Žemdirbystė=Agriculture, vol. 99, No. 1 (2012), p. 85–92
UDK 634.22:633.63:632.3

Slyvų, cukrinių runkelių ir pomidorų svetimkraščių virusų aptikimas bei molekulinė identifikacija

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Santrauka

Svetimkraščiais kaulavaisių ir daržovinių kultūrų virusais yra laikomi virusai, kurie anksčiau Lietuvoje nebuvo pastebėti arba aptikti, yra kilę iš kitų geografinių ar klimato zonų ir turi tendenciją plėsti pažeidžiamų augalų spektrą. Tokiais virusais laikytini: slyvų raupligės virusas *Plum pox virus* (PPV), pažeidžiantis slyvas *(Prunus domestica* L.) ir kitus kaulavaisius, runkelių nekrotinio gyslų pageltimo virusas *Beet necrotic yellow vein virus* (BNYVV), pažeidžiantis cukrinius runkelius (*Beta vulgaris* var. *saccharifera* L.), ir kiauliauogių mozaikos virusas *Pepino mosaic virus* (PepMV), pažeidžiantis pomidorus (*Lycopersicon esculentum* Mill.). Nors iš pietinių Europos kraštų įvežtuose pomidoruose PepMV jau buvo aptinkamas beveik prieš dešimtmetį, Lietuvoje šiltnamiuose išaugintuose pomidoruose šis virusas aptiktas tik prieš porą metų. Virusai identifikacijai buvo išskiriami iš natūralių infekcijos šaltinių, testuojami imunofermentiniais metodais, elektronine mikroskopija, pernešami į žolinius augalus indikatorius. Iš slyvų išskirti izoliatai sukėlė būdingus požymius *Chenopodium foetidum* ir *Pisum sativum* augaluose, BNYVV izoliatai iš rizomaniškų cikrinių runkelių šaknų sukėlė specifinę reakciją *Chenopodium quinoa*, *C. amaranticolor* ir *Tetragonia expansa* augaluose. Į PepMV infekciją tipiškai reagavo inokuliuoti pomidorai ir *Nicotiana* genties augalai indikatoriai. Preliminariai identifikuotų PPV, BNYVV ir PepMV izoliatų identiškumas atitinkamų rūšių virusams buvo patvirtintas DAS-ELISA testais, virionų morfologijos tyrimais elektroniniu mikroskopu, molekulinėmis polimerazinės grandininės reakcijos procedūromis. RT-PGR analizė parodė, kad visi PPV izoliatai priklauso PPV-D kamienų (padermių) grupei, visi BNYVV izoliatai priskirtini B tipo kamienų grupei. Slyvų raupligės virusas ir runkelių rizomanijos sukėlėjas Lietuvoje nėra plačiai paplitę, aptikti tik keliuose rajonuose tam tikrose vietovėse. Kiauliauogių mozaikos virusas aptiktas trijuose gamybiniuose šiltnamiuose. Tam turi įtakos ir augalų apsaugos tarnybos profilaktinė veikla bei taikomos profilaktinės priemonės.

Reikšminiai žodžiai: slyvų raupligės virusas, *Prunus domestica*, runkelių gyslų nekrotinio pageltimo virusas, *Beta vulgaris* var. *saccharifera*, kiauliauogių mozaikos virusas, *Lycopersicon esculentum*, identifikacija, RT-PGR.