Table 4. **The distances between positions of similar nucleotide sites in genomes of two potexviruses**

Site length	Pos1*	Pos2**	Distances, nucleotides	Distances, triplets
62	815	834	19	6,3
72	1896	2704	808	269,3
65	2084	2898	814	271,3
80	2277	3091	814	271,3
60	2442	3262	820	273,3
259	2789	3612	823	274,3
344	3394	4217	823	274,3
69	4953	5820	867	289,0
65	4207	5080	873	291,0

*Pos1 - positions of similar nucleotide sites in genome of foxtail mosaic virus

**Pos2 - positions of similar nucleotide sites in genome of Scallion virus X

Table 5. **Displacement of the distances between positions of similar nucleotide sites in genomes of tobamo- and potyviruses**

Virus pair	No. of similar sites			
	All	with displacement		
		0	1	2
Tobamovirus genus				
Tobacco mild green mosaic virus / Cucumber green mottle mosaic virus	19	0	17	2
Pepper mild mottle virus / Ribgrass mosaic virus	14	2	8	4
Tomato mosaic virus / Crucifer tobamovirus	16	1	3	12
Cucumber fruit mottle mosaic virus / Odontoglossum ringspot virus	20	2	4	14
Potyvirus genus				
Potato virus A / Potato virus Y	19	0	17	2
Scallion mosaic virus / Sugarcane mosaic virus	18	1	16	1
Bean common mosaic virus / Cocksfoot streak virus	17	17	0	0
Soybean mosaic virus / Yam mosaic virus	16	1	14	1

* Displacement is equal to: or integer number of triplets (3x nucleotides, displacement 0), or 3x+1 nucleotides (displacement 1), or 3x+2 nucleotides (displacement 2)

Overall, we tested a new method for the computational search of the similar nucleotide sites in genomes by successive disposition of two genomic sequences with increasing displacement of their initial positions and revealing in all dispositions the similar nucleotide sites, which length and persentage of sequence identity were not less then preliminary user-specified.

We have determined a parameter limits of non-random nucleotide coincidence in sequences as well as the presence and localisation peculiarity of similar nucleotide sites in viral genomes. Our results also demonstrate that the lisianthus necrosis virus is the more closely related to tombusviruses then to necroviruses.

The computational search used provides an alternative sequence similarity analysis without a time-consuming aligning procedure. The method can be used as a simple, fast and obvious way for comparing sequence relatedness with graphical visualization of the similar nucleotide or aminoacid sites.

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INCIDENCE OF PLUM POX VIRUS IN LITHUANIA AND UKRAINE

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According to the internationally agreed joint project "Investigation of distribution of plum pox virus in Lithuania and Ukraine and development of biotechnological methods of obtaining of virus-free planting material" 95 samples of plum, apricot and peach from several locations of Ukraine were collected in June 2007 and checked for the presence of PPV by DAS-ELISA. Presence of PPV was detected in all explored regions. Four isolates PPV from plum of Kyivsky and Transkarpathian regions were transferred to herbaceous plants and the presence of the virus in these plants was confirmed by DAS-ELISA and IEM investigation. The presence of PPV in samples from Lithuania was confirmed in 2 cases from 20. PPV positive samples were collected from Jonava and Sakiai regions.

Introduction. Stone fruit trees are natural hosts of several widespread viruses. Thirty virus diseases of stone and pome fruits have been described in former Yugoslavia [Šutič et al., 1999]. Plum pox virus or 'Sharka' disease agent *Plum pox potyvirus* (PPV) originally described in Bulgaria on *Prunus domestica* gradually has spread throughout European borders [Šutič et al., 1999]. PPV infects virtually all cultivated fruit tree species of the genus *Prunus*. The disease also affects some wild *Prunus* species, especially blackthorn (*Prunus spinosa*) 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

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pox is the most serious disease of plums, apricots and peaches in Europe and is considered as quarantine pathogen in all continents [Roy, Smith, 1994; 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 disease. In some cases, only part of the infected tree or even only a single branch exhibit symptoms. Disease symptoms are most noticeable during spring 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. The disease may not be visible until several months or years after the tree has been infected. Signifficant progress investigating PPV has been made after the discovery of possibility to transmit it to herbaceous plants *Chenopodium foetidum* and *Nicotiana clevelandii* [Sutic, 1961]. PPV was the first plant virus for which serological ELISA technique was created and adopted for detection in 1977 [Clark, Adams, 1977]. Using ELISA technique it was established that beginning of vegetative period when leaves are still not completely expanded virus concentration is highest and it is most suitable time for mechanical virus trasmission. With aging of leaves virus concentration gradually decreases and late in summer the virus is not detectable with electron microscope [Калашян, 1978]. PPV belongs to the most numerous filamentous mechanically and by aphids transmissible plant virus group – potyviruses. PPV virions are filamentous particles c. 660-770x12,5-20 nm [Klinkowski, 1968; Kegler, Schade, 1971]. According to electrophoretic mobility and serological properties most European isolates of PPV belong to two groups of strain – M (Marcus) and D (Dideron) [Bousalem et al., 1994; Cambra et al., 1994; Myrta et al., 1996]. Strains of D group are less aggressive and have limitted host range.

The main source of the virus is infected trees or budwood [OEPP/EPPO, 1983]. From these, PPV is transmitted either by grafting or by aphids in a non-persistent, stylet-borne manner to uninfected hosts. Furthermore, the virus can survive in the roots of rogued infected trees and spread from there by natural root grafting. The aphids acquire the virus during feeding and then transmit it to nearby healthy plants. The aphids can only transmit PPV for a short period of time after acquiring it. Long distance spread of PPV occurs through the movement and use of infected propagative materials (grafting and budding of infected material). The use of disease-free propagative material at all times is fundamental to preventing introduction to new areas. Measures to reduce the significance of plum pox in areas where it is present include the use of disease-free planting stock when planting new orchards or replenishing existing ones, removal of infected trees (including their roots), use of tolerant or less susceptible species or cultivars where possible, proper spacing of trees to reduce the rate of spread, control of aphids to slow spread, and weed control (including wild *Prunus* species) to eliminate reservoirs of either aphids or PPV [Anonymus, 1997; Nemeth, 1994; OEPP/EPPO, 1992].

First evidence for the presence of PPV in Lithuania was received in Lithuanian Institute of Horticulture by Dr. J. Stankiene who using ELISA reagents for PPV (Poland Institute of Horticulture, Skierniewice) detected the presence of the virus in some plum trees from Kaunas region in about 1995. Local isolate of PPV was transmitted to herbaceous plants in 1997 and the specific antiserum in the Institute of Botany was prepared. The Lithuanian isolate (PPV-971) appeared to be attributed to D group of strains, as was identified using monoclonal antibodies in ELISA tests conducted by Dr. D. Boscia (Centro di studio sui virus e le virosi delle colture de Bari, Italy). For molecular diagnosis and characterization of the isolate PPV-971, RT-PCR amplification of the coat protein encoding sequence by use of specific primers complementary to 5'- and 3' parts of PPV coat protein L1 was employed (GenBank accession number X81081). The specific DNA fragment, corresponding to predicted coat protein sequence size, was cloned into *E. coli* pUC57 for DNA sequencing. Amino acid sequence comparison with GenBank data indicated 98.2 % similarity with coat protein of PPV potyvirus isolated by E.Mais et al. (accession no. X81083) and 97.3 % with PPV strain Rankovic [S.Lain et al., 1989; Staniulis et al., 1998; Dargeviciute et al., 2000].

More extensive surveys on spread of PPV using ELISA were carried out after establishment of Phytosanitary Research Laboratory at Lithuanian State Plant Protection Service in 1997. Surveys for determination of PPV incidence during 1998-2002 were carried out in 14 localities of 9 administrative regions of Lithuania. Presence of PPV was confirmed in 865 tree samples of 1553 analysed by ELISA and in 66 tree samples from 150 analysed by ISEM. In 5 outbreaks of the virus all the plum trees were uprooted and burned, and in the other 9 orchards only the contaminated trees and the neighbouring trees were uprooted and burned. During 2003 7,5 hectares of plum trees were surveyed. From 91 samples checked, 9 appeared PPV positive, among them in 2 new locations. Also 206 apricot, 204 peach and 5 cherry trees were surveyed and part of them were checked for PPV presence, however with negative results for the virus. In 2004 32 samples from total 3 ha area of plum trees were checked, and 14 positive samples were detected. Of 1042 checked samples from 5873 apricot tree and 51 samples of 455 peach trees all appeared to be PPV negative. In 2005 235 samples from surveyed 6680 plum trees were checked. 3 positive samples detected were from previously defined locations. In 2006 18 hectares of stone fruit orchards were checked and two PPV infection focus were revealed. The status of PPV in Lithuania can be described as: present only in some areas.

Sharka in Ukraine was detected for the first time in 1966 in the Chernivetsky region, the size of the harmed area was 0,3 hectares. In 1970 Sharka was detected in six regions of Ukraine (Vinnitsky, Transkarpathian, Ivano-Frankivsky, Lvivsky, Ternopilsky and Chernivetsky regions with a general size of the harmed area around 57,58 hectares). The nidus of the infection in the Ivano-Frankivsky (0,105 hectares) and in the Vinnitsky (4,6 hectares) regions was timely eliminated. In spite of hard actions against the harmed area, Sharka has spread in the South (Odesky region) and in the West (Transkarpathian region) of Ukraine. Up to 1.01.2007 the biggest area of stone fruit plantings harmed with PPV was registered in the Transkarpathian region. The size of the area was around 4487,2 hectares. The harmed area in the Odesky region has decreased from 10,5 hectares to 1,5 hectares due to quarantine removal from plantings in the Ovidiopolsky district. It should be mentioned however that investigations for the PVP presence were held using visual diagnostics without confirmation of viral etiology of the disease.

For this investigation leaf samples with suspectable plum sharka disease symptoms were collected in harmed regions of Ukraine and Lithuania for laboratory analysis by DAS-ELISA, immunosorbent electron microscopy (ISEM) and for transmission to herbaceous plants.

Materials and methods. *Stone fruit tree leaf samples indicating virus-like symtoms or symptomless from Ukraine were collected in Kyivsky, Odesky and Transkarpathian regions. From Lithuania plum tree leaf samples were taken in Kaunas, Varena, Trakai, Sakiai and Jonava regions. The survey of incidence of PPV in Lithuania was performed during June in plum tree orchards in expected foci of the infection and field samples were collected. Collected field samples were assayed by DAS-*

ELISA with PPV polyclonal antibodies (BIOREBA, Switzerland or LOEWE, Germany). DAS-ELISA procedures were performed as described by Clark and Adams [1977]. In BIOREBA AB kits polyclonal antibodies were prepared against PPV isolate from the Netherlands reacting with a broad spectrum of PPV isolates from France, Greece and Switzerland. The LOEWE kits contained PPV broad range artificial mixture of antisera. Enzymatic reaction was evaluated photometrically at 405 nm using Labsystems Multiskan RC microtiter plate reader. When the absorbance values in DAS-ELISA tests were not clear-cut enough or near to the critical values of background the plum tree samples were subjected to additional analysis by immunosorbent electron microscopy (ISEM) in Plant Virus laboratory of the Institute of Botany [Derrick, 1973; Brlansky, Derrick, 1979; Kerlan et al., 1981]. For ISEM procedure antibodies prepared against Lithuanian isolate of PPV with homologous titre 1:1024 in microprecipitation reactions were used. For sensibilization of electron microscopic grids before antigen application diluted 1:50 fraction of IgG antibodies was used. Leaf tissue 1:20 (v/w) in extraction buffer (0,05M phosphate bufer pH 7,2, 0,01M DIECA, 2% PVP) clarified by centrifugation at 10 000g for 5 min. was applied as antigen. After IgG and Ag application the grids were subjected to washing by PBS-Tween-20 (0,05%). As a staining solution 2 % uranyl acetate was used. Electron microscopic preparations were examined with JEOL JEM-100S transmission electron microscope at instrumental magnification 10000-25000^{x}. For the mechanical virus transmission to herbaceous plants (Chenopodium feoetidum Schrad., Pisum sativum L. and some Nicotiana L. species) phosphate buffer solution 0,03M containing 1,5 % nicotine, 0,25 % Na DIECA and 0,2 % of Na_2SO_3, final pH 8,0 was used. For the PCR tests RNA extraction from plum leaves or experimentally infected herbaceous plants was carried out according to the instruction of "QuickPrep^{TM} Total RNA Extraction Kit for the direct isolation of total RNA from most eukaryotic tissues or cells" (Amersham Biosciences, UK). For general PPV detection by RT-PCR amplification of a 243 bp fragment in the coat protein (CP) coding region was carried out using the primers P1 and P2 according Wetzel et al. [Wetze et al, 1991]: P1: 5'-3' ACC GAG ACC ACT ACA CTC CC and P2: 5'-3' CAG ACT ACA GCC TCG CCA GA. Temperatures used for the cycling reaction were as follows: 30 min at 42^{0}C; 3 min at 93^{0}C, followed by 40 cycles of 30 sec at 94^{0}C, 30 sec at 54^{0}C, 30 sec at 72^{0}C; with a final extension of 5 min at 72^{0}C [Sertkaya et al., 2003].

***Results.** Typical symptoms of PPV affected plum leaves consisted of mosaic, ring pattern, vein banding with slight deformation of lamina (Fig.1), however symptoms often varied in great extent depending on many factors. Considerable part of analysed samples appeared as PPV negative.*

Fig. 1. Typical symptoms of PPV affected plum (*Prunus domestica*) leaf.

Application of antibodies for sensibilization EM grids very significantly increased quantity of captured virus particles in about 2 orders of magnitude [Brlansky, Derrick, 1979]. Without antibody coating PPV particles are difficult to be detected. Presence of specific virus particles by ISEM can be proved very evidently (Fig. 2).

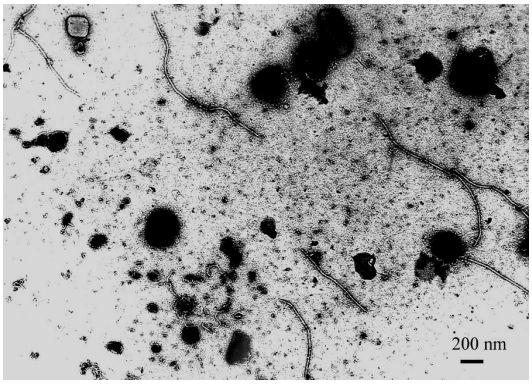

Fig. 2. PPV particles trapped in ISEM preparation from plum leaf extract. Bar represents 200 nm.

Results of tests for detection of PPV by DAS-ELISA in plum tree samples collected in some regions from Lithuania are presented in table 1 and indicate that the virus was present in two localities of Kaunas and Jonava regions.

Table 1. **ELISA test results of Lithuanian plum samples collected in June 2007.**

Tree species and location	No. samples tested	ELISA positive	ISEM positive
Garden plum – Sakiai reg., Patasyne	5	1	1
Garden plum - Trakai reg., Trakai	1	0	0
Garden plum - Varena reg., Perloja	1	0	0
Garden plum - Kaunas reg., Ringaudai	4	0	0
Garden plum - Jonava reg., Pedziai	6	1	1

Samples of stone fruite cultures collected in 3 regions were further analyzed via ELISA for PPV antigens. This study resulted in demonstration of Sharka disease in every region investigated, despite the differences in the percentage of infected plants (Fig 3).

From 40 samples tested for the Transkarpathian region, PPV has been detected in 19, which makes 45,7 % of disease rate. The virus was detected in 6 districts from 8 investigated. Regularly, PPV was observed on plum (*Prunus domestica*) and apricot (*Prunus armeniaca*), this correlates with the outcomes of visual diagnostics.

The disease rate for 2 districts in the Odesky region was shown to be 32% (10 PPV-positive samples from 31 tested). Interestingly, in in the Odesky region PPV was detected on plums, apricots and peaches.

In the Kyivsky region, the disease rate was at 13%; the virus has been detected in one district in one of three investigated plum samples (*Prunus domestica*).

Mechanical transmission to the herbaceous plants was conducted from plum leaf samples positively reacted in DAS-ELISA tests using phosphate buffer with antioxydant substances. From all ELISA positive samples inoculated *Pisum sativum* plants developed vein clearing (VC) and slight mottling (Mo) systemic symptoms (Fig. 4). During initial inoculations *Chenopodium foetidum* plants did not develop noticeable local lesions (LL) (table 2). Slight chlorotic local lesions usually appeared after few additional reinoculation. ELISA tests and electron microscopy confirmed presence of PPV in experimentally inoculated herbaceous plants.

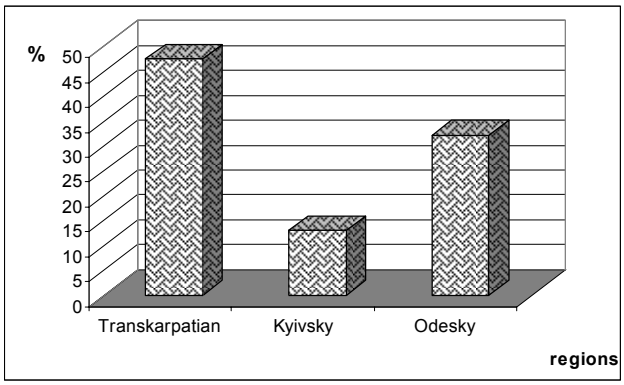

Fig. 3. Percent of PPV infected plants with positive result obtained by ELISA from different regions of Ukraine.

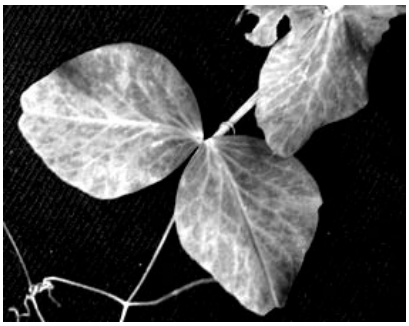

Fig. 4. Systemic mottle of leaf *Pisum sativum* infected by PPV.

Table 2. **Reaction of experimentally inoculated plants with leaf extracts from ELISA positive plum tree samples.**

Plant species	Symptoms	ELISA test results
Chenopodium foetidum	LL	+
C. quinoa	-	-
Nicotiana benthamiana	slight mottling	-
N. debneyi	-	-
Pisum sativum cv. 'Peliuškos'	VC, Mo	+
P. sativum, cv. 'Rainiai'	VC, Mo	+
P. sativum, cv. 'Žalsviai'	VC, Mo	+

The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers [*Wetzel, T.* 1991]. PCR reaction of all tested samples resulted in specific amplification of 243 bp fragment of the coat protein region (Fig. 5). No products were obtained from negative control sample.

Thus, we demonstrated rather high percentage of *Plum pox virus*-infected stone fruit cultures in Ukraine. The status of PPV in Lithuania can be described as: presently just in some areas. From 20 samples of plum trees collected in Lithuania the presence of PPV was confirmed by DAS-ELISA in two cases from the Jonava and Sakiai regions. Morphology of the viral parts was investigated. Four Ukrainian isolates of PPV from the plums in the Kyivsky and Transkarpathian regions were propagated on herbaceous indicator plants. The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers.

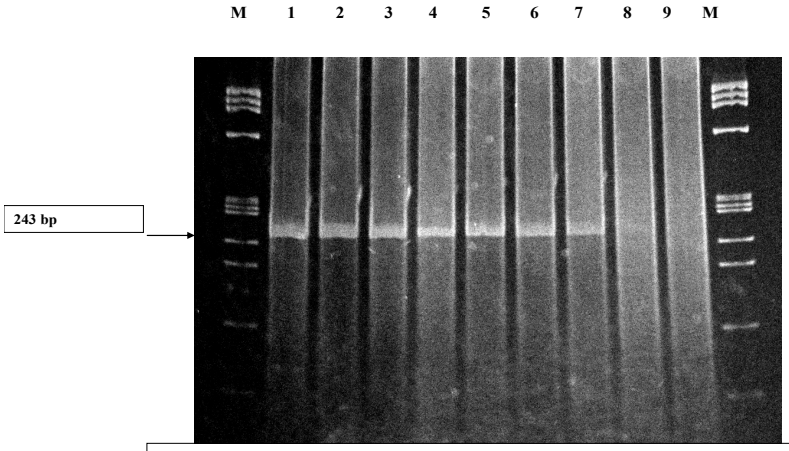

Fig. RT-PCR of PPV specific 243 bp fragment. Lanes M – DNA-ladder [*ΦX 174 DNA/BsuRI (Hae III)*], lanes 1 and 2 – Lithuanian plum samples, lanes 3-8 – Ukrainian plum samples, lane 9 – control (healthy plant). Samples: 1. Patasyne, 2. Pedziai, 3. Novosilki N3, 4. Zuliany, 5. Novosilki N4, 6. Uzgorod N1, 7. Uzgorod N6, 8. Uzgorod N11.

Fig. 5. RT-PCR of PPV specific 243 bp fragment.

Thus, we demonstrated rather high percentage of *Plum pox virus*-infected stone fruit cultures in Ukraine. The status of PPV in Lithuania can be described as: presently just in some areas. From 20 samples of plum trees collected in Lithuania the presence of PPV was confirmed by DAS-ELISA in two cases from the Jonava and Sakiai regions. Morphology of the viral parts was investigated. Four Ukrainian isolates of PPV from the plums in the Kyivsky and Transkarpathian regions were propagated on herbaceous indicator plants. The presence of PPV in plum leaves tissue and indicator plants was proved by RT-PCR, using general primers.

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BEET NECROTIC YELLOW VEIN VIRUS: PURIFICATION AND DETECTION BY ELECTRON MICROSCOPY AND WESTERN BLOT

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The best results for Beet necrotic yellow vein virus (BNYVV) purification were obtained from Chenopodium quinoa leaf tissue mechanically inoculated with the virus using slightly modified method described by Bouzoubaa [1]. The presence of BNYVV was confirmed by immunosorbent electron microscopy (IEM) and Western blot analysis. IEM with antiserum to BNYVV coat protein (CP) revealed virus particles morphology typical for rhizomania agent [8]. Purified virus suspension was used for CP evaluation and detection by Western blot.

Introduction. BNYVV (genus *Benyvirus*) causes rhizomania – destructive disease of sugar beet (*Beta vulgaris* L. ssp. vulgaris). BNYVV is rigid, rod-shaped virus, composed of particles 390, 265, 100 and 85 nm long and 20 nm wide [8]. The multipartite single-stranded RNA genome consists of four RNA species, and the 5th species is being found in some isolates (6.8, 4.7, 1.8, 1.5 and 1.45 kb) [10]. The virus is transmitted by vector, the soilborne plasmodiophorid *Polymyxa betae* Keskin, which survives in infested soil for many years [11]. The disease now has a worldwide distribution (Europe, Asia and North America) [9] and is characterized by reduced taproot size with extensive proliferation of lateral rootlets that leads to decline in root yields and sugar content [6].

Surveys to detect the presence of BNYVV have been regularly carried out in Lithuania since 1998 using DAS-ELISA test. In recent years this virus was detected in two areas of Lithuania [3, 13]. *C. quinoa* plant tissues mechanically inoculated with crude extract of infected sugar beet rootlets were used for investigation of rhizomania agent. Here we present identification of BNYVV using EM (i.e., the particle morphology) and Western blot assay (i.e., CP molecular weight).

Materials and methods. Isolation of BNYVV was performed by mechanical inoculation of *C. quinoa* using infected sap of sugar beet bearded roots. Symptomatic leaf tissue was tested for BNYVV by DAS-ELISA [2] using DSMZ immunological kit, for virus particles morphology by EM and further it was used for virus purification.

BNYVV was purified as described by Bouzoubaa with some modification [1]. Healthy *C. quinoa* extract was used as negative control. The virus was concentrated twice by PEG, NaCl. Afterwards the virus was purified by high speed ultracentrifugation through 20 % sucrose cushion and in 5-45 % sucrose density gradient centrifugation. Purified virus suspension was used for EM examination and Western blot analysis.

In attempts to examine virus particles, carbon coated grids were placed on drops of purified virus suspension, stained with 2% uranyl acetate (UA), and were viewed using JEOL JEM-100S electron microscope at x 20 000 magnification.

For BNYVV protein analysis purified virions were denatured and CP was separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) in duplicate [5]. One gel was stained with PageBlue™ Protein Staining Solution. Capsid molecular mass was estimated using PageRuler™ Prestained Protein Ladder. For Western blot analysis the other gel with BNYVV CP was transferred to nitrocellulose membrane [12]. Membrane was probed with IgG to BNYVV (diluted 1:250) and the bound IgG was detected with secondary antibody conjugated to alkaline phosphatase (diluted 1:1000). Antibodies used for Western blot analysis were the same as supplied for DAS-ELISA kit. Blot was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

Results and Conclusions. IEM investigation of infected sugar beet rootlets revealed the presence of characteristic different lengths BNYVV particles about 20 nm in diameter (Fig. 1) [8, 10]. Tissue extracts from these rootlets was used for mechanical inoculation of indicator plant - *C. quinoa*, which leaves developed local chlorotic lesions after 5-7 days (Fig. 2).

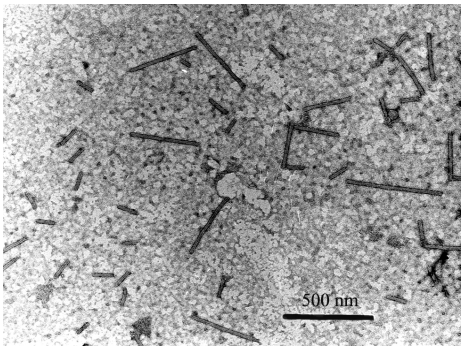

Fig. 1. IEM (instrumental magnification 20 000 x): BNYVV characteristic virus particles about 20 nm in diameter from extracts of sugar beet rootlets.

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