VILNIUS UNIVERSITY
INSTITUTE OF BOTANY OF NATURE RESEARCH CENTRE

Genovaitė Marija Žižytė

THE IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF SUGAR BEET RHIZOMANIA CAUSING VIRUS

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Scientific Supervisor:

Habil. dr. Juozas Benediktas Staniulis (Institute of Botany, Nature Research Centre, biomedical sciences, biology – 01B, microbiology, bacteriology, virology, mycology – B 230)

Consultant:

Prof. Jari Valkonen (University of Helsinki Department of Agricultural Sciences, Finland)

The doctoral dissertation will be defended at Vilnius University Biology Science Council:

Chairman:

Prof. dr. Donaldas Čitavičius (Vilnius University, biomedical sciences, biology – 01B, microbiology, bacteriology, virology, mycology – B 230)

Members:

Dr. Audronė Valerija Kalvelytė (Institute of Biochemistry, physical sciences, biochemistry – 04P)

Prof. habil. dr. Aniolas Sruoga (Vytautas Magnus University, biomedical sciences, ecology and environmental science – 03B)

Dr. Vaidotas Lygis (Institute of Botany Nature Research Centre, biomedical sciences, biology – 01B, microbiology, bacteriology, virology, mycology – B 230)

Dr. Deividas Valiūnas (Institute of Botany Nature Research Centre, biomedical sciences, biology – 01B, microbiology, bacteriology, virology, mycology – B 230)

Opponents:

Assoc. prof. dr. Juozas Raugalas (Vilnius University, biomedical sciences, biology – 01B, microbiology, bacteriology, virology, mycology – B 230)

Dr. Irena Brazauskienė (Institute of Agriculture Lithuanian Research Centre for Agriculture and Forestry, biomedical sciences, agronomy – 06 B, crop sciences, horticulture, plant protection, phytopathology – B 390)

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Address: Žaliųjų Ežerų 49, LT-08406, Vilnius, Lithuania

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Genovaitė Marija Žižytė

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Mokslinis vadovas:

Habil. dr. Juozas Benediktas Staniulis (GTC Botanikos institutas, biomedicininiai mokslai, biologija – 01B, mikrobiologija, bakteriologija, virusologija, mikologija – B 230)

Konsultantas:

Prof. Jari Valkonen (Helsinkio universitetas, Žemės ūkio mokslo departamentas, Suomija)

Disertacija ginama Vilniaus universiteto Biologijos mokslo krypties taryboje:

Pirmininkas:

Prof. dr. Donaldas Čitavičius (Vilniaus universitetas, biomedicininiai mokslai, biologija – 01B, mikrobiologija, bakteriologija, virusologija, mikologija – B 230)

Nariai:

Dr. Audronė Valerija Kalvelytė (Biochemijos institutas, fiziniai mokslai, biochemija – 04P)

Prof. habil. dr. Aniolas Sruoga (Vytauto Didžiojo universitetas, biomedicininiai mokslai, ekologija ir aplinkotyra – 03B)

Dr. Vaidotas Lygis (GTC Botanikos institutas, biomedicininiai mokslai, biologija – 01B, mikrobiologija, bakteriologija, virusologija, mikologija – B 230)

Dr. Deividas Valiūnas (GTC Botanikos institutas, biomedicininiai mokslai, biologija – 01B, mikrobiologija, bakteriologija, virusologija, mikologija – B 230)

Oponentai:

Doc. dr. Juozas Raugalas (Vilniaus universitetas, biomedicininiai mokslai, biologija – 01B, mikrobiologija, bakteriologija, virusologija, mikologija – B 230)

Dr. Irena Brazauskienė (Žemdirbystės institutas Lietuvos agrarinių ir miško mokslo centro filialas, biomedicinos mokslai, agronomija – 06 B, augalininkystė, sodininkystė, augalų apsauga, fitopatologija – B 390)

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Introduction

Over 50 diseases are known to affect sugar beets worldwide, of which nearly 20 have economic importance (Mukhopadhyay, 1987). European sugar beet (*Beta vulgaris* subsp. *vulgaris* convar. *vulgaris* var. *altissima* Döll) crops are affected by several different groups of diseases caused by viruses. Symptoms caused by yellowing viruses, belonging to the families *Luteoviridae* and *Closteroviridae*, occur mainly in leaves, causing varying degrees of chlorosis. These viruses are transmitted by aphids (Stevens et al., 2005b). Thirty soilborne viruses or virus-like agents are transmitted by fungal vectors (Campbell, 1996). Of these, severe sugar beet yield losses are due to rhizomania agent transmitted by the obligate root-infecting parasite *Polymyxa beta* Keskin (Tamada and Baba, 1973). Rhizomania is a soil-borne disease that occurs throughout the major sugar beet growing regions of the world, causing severe yield damage in the absence of effective control measures. It is caused by *Beet necrotic yellow vein virus* (BNYVV). BNYVV has a multipartite RNA genome with all natural isolates containing four RNA species, although some isolates have a fifth RNA (Tamada et al., 1989). RNA1 and RNA2 contain the housekeeping genes of the virus and are required for infection, whereas the smaller RNAs are involved in pathogenicity and vector transmission (Richards and Tamada, 1992).

On the basis of restriction fragment length polymorphism (RFLP), single strand conformation polymorphism or sequence analysis of RT-PCR products from RNA1-5, three major (A, B and P) types of BNYVV have been identified, which can not be distinguished serologically (Kruse et al., 1994; Koenig et al., 1995; Ratti et al., 2005). The A type usually is present in most European countries, Iran, N. America, China and Japan, whereas the B type is detected frequently in France, Germany and in some cases in Sweden, China and Japan (Saito et al., 1996; Miyanishi et al., 1999; Lennefors et al., 2000; Sohi and Maleki, 2004). The sequence divergence between BNYVV A and B type is low, in the range of 96-99 % (Saito et al., 1996, Koenig and Lennefors, 2000). Although it has been suggested that there is no difference in pathogenicity between the two types it was shown that the BNYVV A type appeared to be slightly more pathogenic than the B type on sugar beet cultivars (Heijbroek et al., 1999, Rush et al., 2006).

The European and Asian isolates containing RNA 5 were designated as P type (Koenig et al., 1997; Koenig and Lennefors, 2000). Such isolates containing RNA 5 were also detected in the United Kingdom, China and Japan (Tamada et al., 1996; Koenig and Lennefors, 2000, Harju et al., 2002). These isolates appeared to be more aggressive and pathogenic than other BNYVV isolates (Tamada et al., 1996; Miyanishi et al., 1999). BNYVV P type moves more rapidly in plants than the A or B types and, in partially resistant cultivars, much higher levels of infection are detected in the tap roots compared with plants infected with the A or B types (Heijbroek et al., 1999). The BNYVV P type may evade the activation of plant defense responses therefore the P type is a considerable potential threat to sugar beet cultivation worldwide where the control of rhizomania is based solely on the use of virus-resistant cultivars (Klein et al., 2007).

Surveys and molecular analysis of BNYVV isolates may help to establish a correlation between molecular properties and the geographical origin of BNYVV. Genome sequence analysis can help to characterize the diversity of isolates, to determine the type of virus and to identify changes in gene-encoded protein sequences, resulting in the BNYVV pathogenicity.

The use of polyclonal antibodies in ELISA methods can be adapted for the routine detection of this virus. This would allow selecting virus isolates for biological and morphological properties determination and virus molecular characterization.

The aim of this work was to characterize the rhizomania causing virus in Lithuania using biological, ELISA, electron microscopy and molecular techniques; to establish molecular type of detected Lithuanian rhizomania agents and to assess phylogenetic relationships of identified BNYVV isolates protein sequences with well-known BNYVV isolates in the other countries; to obtained the polyclonal antibodies against BNYVV from the rabbit and to investigate the possibility of using antibodies for the diagnosis of this virus.

The tasks of this work were as follows:

1. To identify rhizomania causing *Beet necrotic yellow vein virus* (BNYVV) in sugar beet crops in Lithuania using ELISA and different PCR methods. To establish the most effective and sensitive PCR method suitable for BNYVV identification.
2. To transmit, propagate BNYVV in indicator plants, and to obtain the pure virus preparation, to identify and compare morphological and serological properties of the virions found in the infection sources and in the indicator plants.
3. To identify the types of detected BNYVV isolates.
4. To compare the fragments of virus isolates coat protein and P25 genes sequences, detected in different geographic areas.
5. To obtain polyclonal antibodies against BNYVV.
6. To prepare the conjugate of BNYVV antibodies with horseradish peroxidase and to assess the suitability of the obtained preparations for the diagnosis of this virus.

Novelty of the research.

- ✓ For the first time *Beet necrotic yellow vein virus* (BNYVV) has been characterized in sugar beet crops in Lithuania using molecular biology techniques.
- ✓ Two different Lithuanian BNYVV isolates types (A and B) were identified. It was found that all Ukrainian BNYVV isolates belong to A type, while the isolate from Kazakhstan – to P type.
- ✓ The morphological and serological properties of this virus were identified.
- ✓ The pure BNYVV preparation suitable for antibody production was obtained. Immunoassay system suitable for diagnosis of BNYVV was developed.

Thus, this research has contributed to the investigation of BNYVV isolates diversity and variability.

The defensive statements:

1. *Beet necrotic yellow vein virus* (BNYVV) was identified in Lithuania, Ukraine and Kazakhstan sugar beet samples with rhizomania symptoms using ELISA and molecular methods.
2. Morphological features of detected virions in rhizomania infected plants and in purified virus preparations are characteristic to BNYVV.
3. Detected Lithuanian BNYVV isolates belong to different virus types: Pn – to A type, while St and T – to B type. It was found that all Ukrainian BNYVV isolates belong to A type, while the isolate from Kazakhstan – to P type.
4. Lithuanian BNYVV isolates contain distinct amino acids tetrads (in 67-70 position) in P25 protein sequence. The correlation between BNYVV isolates coat protein and P25 was shown: Lithuanian St and T isolates belong to B type CP isolates with uniform AYHR tetrad motif, Pn – to A type CP isolates with variable tetrad motif.
5. The obtained purified BNYVV preparation is suitable for polyclonal antibody production.
6. Immunoenzyme system consisted of the obtained polyclonal antibodies against BNYVV and conjugate of polyclonal antibodies and horseradish peroxidase is suitable for diagnosis of BNYVV using ELISA and Western blot methods.

Approbation of results. The dissertation material was reported on 5 international conferences and 2 conferences in Lithuania. The results of the research were presented in 4 scientific articles and in 7 abstracts of conferences.

Structure of dissertation. The dissertation consists of the following chapters: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, References (204 sources), List of publications. The dissertation covers 125 pages; it contains 26 figures and 14 tables. The text of the dissertation is written in Lithuanian with the abstract in English.

Materials and Methods

Virus mechanical inoculations. BNYVV was mechanically inoculated on test and indicator plants recognized as diagnostic species for the BNYVV. For this purpose, carborundum powder was dusted on the plant leaves before inoculation. Leaves of young indicator plants (in 3-5 leaves stage) were inoculated by rubbing their surface with the inoculum of buffered infected plant sap. Local chlorotic or semi-necrotic lesions developed on the indicator plants after 3-7 days, depending on the temperature. The maximum amount of virus in leaf tissue was concentrated within 4-7 days. In the case of a systemic infection, symptoms appeared within a week or more after inoculation. After this period, the leaves with clearly noticeable symptoms were collected, weighted, labeled and stored at minus 2° C.

Virus purification. Locally infected indicator plants (*Chenopodium quinoa, C. amaranticolor*, *Tetragonia expansa*) were used for maintenance of BNYVV. The best results of BNYVV purification were obtained from infected *C. quinoa* leaf tissue using slightly modified

method described by Bouzoubaa (1998). The virus was concentrated twice by PEG, NaCl and 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, for polyclonal antiserum production and for detection of coat protein (CP) by Western blot analysis.

Polyclonal antiserum production. Freshly purified BNYVV preparation was used for the production of polyclonal antibodies in rabbit. The viral suspension (0.34 mg) emulsified with Freund's complete adjuvant (1:1, v/v) was injected at multiple sites under rabbit skin. The second injection (0.2 mg virus suspension) with Freund's incomplete adjuvant (1:1, v/v) was injected intramuscularly after three weeks. The last injection (0.05 mg virus suspension plus saline without adjuvant) was given at interval of one week into the vein of the rabbit ear. The rabbit was bled 3 days after the last injection. The blood was allowed to coagulate at room temperature for 2 h and then kept at 4° C overnight. The antiserum separated from blood clot was centrifuged at 5,000 g for 15 min. Immunoglobulins were purified by saturated ammonium sulphate precipitation, mixed with sterile glycerol (1:1 ratio, v/v) and stored at -20° C in small aliquots.

HRP-conjugate preparation. Horseradish peroxidase (Merck) was conjugated to polyclonal antibodies against BNYVV (pAb-BNYVV) using slightly modified method of G. B. Wisdom (2004). 5 mg HRP was dissolved in 1 ml water, 0.25 ml 0.2 M sodium periodate was added and the solution was incubated for 20 min at room temperature in the dark. After that the solution was eluted through Sephadex G-25 column in 1 mM sodium acetate buffer (pH 4.5) and added to pAb-BNYVV solution. The pH of obtained solution was adjusted to 9.5 by adding 1/10 volume of 0.2 M sodium carbonate buffer (pH 9.5). The mixture was incubated for 2 h at room temperature in the dark. The weight ratio of HRP and antibody was l/l. For reduction 0.2 ml of 4 mg/ml freshly prepared sodium borohydride (Roth) was added and the solution was incubated for 2 h at 4° C. After overnight dialysis against PBS, BSA (Serva) and glycerol (Roth) were added to give a final concentration 2 % and 50 % respectively. HRP-conjugates were stored at -20° C.

Indirect ELISA. Indirect ELISA was used to estimate the titer of pAb-BNYVV. ELISA was carried out in 96-well microtiter plates (Nunc, Denmark). 50 µl solution of antigen (5 µg/ml) in immobilization buffer (0.05 M sodium carbonate, pH 9.5) was loaded into each well of the ELISA plate. Plate was incubated overnight at 4° C. The wells were washed with 200 µl/well ELISA wash buffer (PBS containing 0.1 % Tween-20) and were blocked with 150 µl of ELISA blocking buffer (PBS containing 1 % albumin) for 0.5 h at room temperature. After washing, the wells were loaded with 100 µl of polyclonal antiserum at various dilutions ranging from 1:200 to 1:102400 in PBS-T buffer. The plate was incubated for 1 h at room temperature. After washing, the secondary antibody goat-anti rabbit conjugated with horseradish peroxidase (HRP) (Bio-Rad) was added at 1:3000 dilution in PBS-T buffer and incubated for 1 h at room temperature. The conjugated enzyme was detected by addition of the TMB ONE (ready-to-use) substrate solution (MBI Fermentas) (100 µl/well) and incubated in the dark at room temperature for approximately 10-15 min until sufficient colour developed. The reaction was stopped with 50 µl 3.6 % sulphuric acid solution. Absorbance values were read at 450 nm using a microtitere plate reader (Tecan, Groedig, Austria). The antibodies titer was considered such antibodies dilution of ELISA at which optical density was equal of one.

Direct ELISA. Direct ELISA was used to estimate the titer of pAb-BNYVV conjugated to HRP. The ELISA procedure was the same as mentioned above except that, after washing plates immobilized with antigen over night, the wells were loaded with 100 µl of pAb-BNYVV HRP conjugate at various dilutions ranging from 1:100 to 1:6400 in PBS-T buffer. The plate was incubated for 1 h at room temperature and washed with PBS-T buffer. The conjugated enzyme was detected the same as in indirect ELISA.

Double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and Triple antibody sandwich-ELISA (TAS-ELISA) were performed using Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) immunological kits as described by Clark and Adams (1997). The ELISA test was negative if absorbance of the investigated sample was less than 3 times the absorbance of the negative (healthy plant) control. The ELISA test was positive if absorbance of the investigated sample was equal or greater than 3 times the absorbance of the negative control.

RNA extraction was performed with QuickPrep total RNA extraction kit for the direct isolation of total RNA from most eukaryotic tissues or cells (Amersham Bioscience) or TRIzol Reagent (Invitrogen) according to the kit protocol.

Western blot. For BNYVV protein analysis purified virus preparations and crude extracts of indicator plants or sugar beet rootlets were denatured and CP was separated by 12 % separating and 4 % stacking slab sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) in duplicate (Laemmli, 1970). One gel was stained with PageBlue™ Protein Staining Solution (MBI Fermentas). For Western blot analysis the other gel with BNYVV CP was transferred to PVDF (polyvinylidene fluoride) membrane. An extract from noninfected *C. quinoa* was included in the transfer, as well as prestained protein ladder. After electrophoresis, the gel was allowed to equilibrate in transfer buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS and 20 % methanol) for 10 min. Gels stained with PageBlue™ Protein Staining Solution (MBI Fermentas) were used to visualize proteins. CP molecular mass was estimated using a 10-170 kilodalton (kDa) PageRuler™ Prestained Protein Ladder (MBI Fermentas). For Western blot analysis the proteins were electrophoretically transferred to PVDF membrane (40 mA) for 40 min.

One transfer was incubated for 30-60 min at room temperature with blocking buffer (PBS with 1 % gelatine), then 1-2 h with pAb-BNYVVV conjugated to HRP diluted in 0.1 % PBS-T and 1 % gelatine buffer (1:300) at room temperature with shaking. The blot was washed with shaking (7-8 times, 5 min per wash) with PBS-T buffer and finally 2-3 times with distilled water. The blot was visualized with TMB liquid substrate system for membranes (Sigma). After staining the membrane was rinsed with distilled water and air-dried.

Other transfer was incubated for 30-60 min at room temperature with blocking buffer (PBS with 1 % gelatine), then 1-2 h with pAb-BNYVVV diluted in 0.1 % PBS-T and 1 % gelatine buffer (1:1000) at room temperature with shaking. The blot was washed 5-6 times with PBS-T buffer 5 min per wash. Anti-rabbit IgG Alkaline Phosphatase conjugate developed in goat (Sigma; 1:30.000 dilution) was used as secondary antibody with shaking 1h at room temperature. The blot was washed with shaking (7-8 times, 5 min per wash) with PBS-T buffer and finally 2-3 times with distilled water. Colour was developed by immersing the blot in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system for membranes (Sigma) at room temperature with shaking. After staining the membrane was rinsed with distilled water and air-dried.

Electron microscopy (EM). In attempts to examine virus particles, carbon coated grids were placed on drops of virus infected leaf suspension (with 0.05 M phosphate buffer, pH 7.4) or purified virus suspension, stained with 2 % uranyl acetate (UA), and were viewed using JEOL JEM-100S transmission electron microscope.

Immunosorbent Electron microscopy (IEM). Carbon coated grids were placed 1 h on drops of antibodies diluted 1:100 in 0.05 M phosphate buffer, pH 7.4. Then grids were washed and test samples were laid down on it. After staining with 2 % UA, samples were viewed using JEOL JEM-100S transmission electron microscope.

BNYVV identification by PCR. Various PCR methods have been applied for rhizomania agent analysis: reverse transcription polymerase chain reaction (RT-PCR), nested PCR (nPCR), multiplex RT-PCR (mRT-PCR), immunocapture RT-PCR (IC-RT-PCR). PCR methods have been used for different purposes: BNYVV detection and identification, determination of BNYVV type, amplification of BNYVV genome specific genes for sequence

analysis and RFLP analysis. In each case, specific PCR primers and reaction conditions (Table 1.) were chosen.

Two step RT-PCR cDNA production was performed using 3 µl of template RNA, 2 µl of reverse primer (20 µM) and 200 units of MMLV reverse transcriptase (MBI Fermentas) in a 20 µl volume at 42° C for 1 h according to the manufacturer's instructions. For each PCR reaction a special PCR mix was prepared (20 µM forward and reverse primers, 10×*Taq* reaction buffer, 25 mM $MgCl_2$, 10 mM dNTPs and 5 u/µl *Taq* DNA polymerase (MBI Fermentas)) and depending on the chosen primers, the specific reaction conditions and different thermocycling temperature regimes were used (Table 1.). PCR amplification was performed in 50 µl reaction volumes containing 46-45 µl PCR mix and 4-5 µl cDNA.

One step PCR was undertaken in 50 µl reaction volumes containing 48 µl special PCR mix (0.2 mM of forward and reverse primers, 10×*Taq* reaction buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 200 u/µl of MMLV reverse transcriptase and 5 u/µl *Taq* DNA polymerase (MBI Fermentas)) and 2 µl of template RNA. Different thermocycling temperature regimes are showed in Table 1.

Conventional RT-PCR (identification of BNYVV CP) was carried out using specific primers (Henry et al., 1995; EPPO Bulletin, 2004) (Table 1.), which amplify a 500 bp fragment of the read-through region of the coat protein gene located on RNA-2 of BNYVV.

nPCR was performed with internal primers (Morris et al., 2001; EPPO Bulletin, 2004) (Table 1.), which annealed to the amplicon, produced from conventional RT-PCR, and specifically amplified a 326 bp product.

For IC-RT-PCR method the pathogen was partially purified by adsorbtion with specific antibodies (DSMZ) on a solid-phase (in microtubes). Then tubes were washed with PBST buffer and extracts like for DAS-ELISA were added to the tubes. Finally RT-PCR reaction mix was added to each sample tube and thermocycled at temperatures which were mentioned in conventional RT-PCR for BNYVV CP (500 bp fragment) amplification (Table 1.).

Table 1. Primers and thermocycling temperature regimes used for PCR detection of BNYVV.

Reaction type	**Primer sequence 5´-3´**	**PCR product, bp**	**Thermocycling temperature regimes**
Identification of BNYVV CP /immunocapture RT-PCR	F: CGATTGGTATGAGTGATTT(A) R: ACTCGGCATACTATTCACT(T)	500	37° C – 30 min; 94° C – 2 min; 30 cycles: 94° C – 1 min; 55° C – 1 mim; 72° C – 1 min; 72° C – 3 min; 4° C – ∞.
BNYVV CP nPCR	F: ATAGAGCTGTTAGAGTCACC R: GACGAAAGAGCAGCCATAGC	326	94° C – 2 min; 30 cycles: 94° C – 1 min; 58° C – 1 min; 72° C – 1 min; 72^0 C – 3 min; 4° C – ∞.
BNYVV CP amplification	F: ATGTCGAGTGAAGGTAGATATATG R: ATTGTCCGGGTGGACTGGTTC	567	94° C – 3 min; 35 cycles: 94° C – 1 min; 61° C – 1 min; 74° C – 1 min; 72° C – 10 min; 4° C – ∞.

BNYVV p25 amplification	F: GTTGTTGTGTTTTCTGATCATCATT R: GTGTTGTTGAAATTGTGATAACTC	719	94° C – 3 min; 35 cycles: 94° C – 1 min; 64° C – 1 min; 72° C – 1 min; 72° C – 10 min; 4° C – ∞.
BNYVV p42 amplification (RFLP analysis)	F: ATGGTCCAAGTACAGC R: TAGACATTATCTATC	1160	94° C – 3 min; 30 cycles: 94° C – 30 s; 54° C – 30 s; 72° C – 2.5 min; 72° C – 7 min; 4° C – ∞.
BNYVV TGB amplification	F: ACCGGAGATACTTTTTGCG R: AACAAAAACACCGCCAAACC	722	37° C – 30 min; 95° C – 5 min; 30 cycles: 94° C – 40 s; 60° C – 40 s; 72° C – 45 s; 72° C – 10 min; 4° C – ∞.
BNYVV B type detection	F: TAATAGTATCACTGTTACAACGATTAAGA R: GTCACCTCTTTTTACCATTATATCAG	324	94° C – 1 min; 45 cycles: 94° C – 20 s; 66° C – 20 s; 72° C – 20 s; 72° C – 10 min; 4° C – ∞.
BNYVV A type detection	F: TTGGGCAGCAACTTA R: ACGGTGAGTACAACATACTGA	178	
Multiplex RT-PCR detection of BNYVV CP	F: ACATTTCTATCCTCCTCCAC R: ACCCCAACAAACTCTCTAAC	545	94° C – 3 min; 35 cycles: 94° C – 30 s; 63° C – 30 s; 72° C – 2 min; 72° C – 7 min; 4° C – ∞.
Multiplex RT-PCR detection of BSBV replicase	F: CTTACGCTGTTCACTTTTATGCC R: GTCCGCACTCTTTTCAACTGTTC	399	
Multiplex RT-PCR detection of BVQ replicase	F: GCTGGAGTATATCACCGATGAC R: AAAATCTCGGATAGCATCCAAC	291	

BNYVV type determination: PCR/RFLP analysis. For RFLP analysis specific primers for p42 gene amplification and specific restriction endonucleases (*Taq*I, *Hinc*II, *Acc*I and *Bst*UI) were used (Kruse et al., 1994). RT-PCR amplified (1160 bp) products were incubated with *Taq*I, *Hinc*II, *Acc*I and *Bst*UI overnight at 37° C. Digested products were analyzed by gel electrophoresis, then stained and visualized under UV light.

Multiplex RT-PCR for BNYVV type determination. For mRT-PCR two stages PCR was used: in the first stage RT-PCR was carried out using specific primers (Ratti et al., 2005) (Table 1.) for TGB gene amplification. In the second stage, nPCR was carried out using previously amplified TGB fragment and A/B type specific primers.

Multiplex RT-PCR for BNYVV, BSBV and BVQ detection. Three pairs of primers (Meunier et al., 2003) (Table 1.) were combined in the mRT-PCR for specific BNYVV, BSBV and BVQ products (545 bp, 399 bp and 291 bp) amplification.

In all PCR methods healthy plant material and water controls were used. All PCR products were separated by electrophoresis in 1-2 % agarose or 5 % bisacrylamide gels, stained with ethidium bromide and visualized under UV light.

Sequencing analysis. For BNYVV CP and p25 amplification specific primers (Schirmer et al., 2005) (Table 1.) were used. Amplicons corresponding to CP (567 bp) and p25 (719 bp) were purified using DNA extraction kit (MBI Fermentas) before sequencing. PCR products were sequenced (Institute of Biotechnology, DNA Sequencing Centre, Lithuania and "Macrogen", Korea) to both directions and were jointed to determine the consensus sequence using the DNASTAR program. Additional sequences for multiple sequence alignments were obtained from gene bank database (Genbank, http://www.ncbi.nlm.nih.gov/). Sequences were analyzed using

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi),

CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html),

ProtParam tool (http://www.expasy.org/tools/protparam.html) and

EMBOSS Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) programs.

The CLUSTAL W (Thompson et al., 1994) was used to perform multiple nucleotide and amino acid sequence alignments. Phylogenetic trees were constructed by MEGA 4.1 (Tamura et al., 2007) using the neighbor-joining method. The significance of branch order was estimated by 1.000 bootstrap replicates.

Results and Discussion

Rhizomania: symptoms, distribution and identification with DAS-ELISA.

Fig.1. Typical symptoms of rhizomania: A – sugar beet leaf with yellow veining and necrosis. **B, C, D** – sugar beet roots with proliferated and partly necrotic fibrous rootlets.

The main symptoms of rhizomania also known as "root madness" include root bearding, stunting, crinkling, wilting, chlorosis of leaves, yellow veining and necrosis of leaf veins. BNYVV is mostly limited to sugar beet roots, and very rarely move into the upper part of the plant, providing yellow veining symptom on the leaves (Tamada et al., 1989). Infected sugar beets with typical symptoms of rhizomania are illustrated in Fig. 1.

The diagnostic root symptoms of rhizomania (stunting, constriction, and extensive root

proliferation) are showed in Fig. 1. B, C, D. Sugar beet leaf with yellow, necrotic veining symptom resulted in the cultivation of infected sugar beet in the greenhouse for several years (Fig. 1. A).

Sugar beet crops in Lithuania covers more than 20 000 Ha. Surveys for the presence of BNYVV in sugar beet crops in Lithuania have regularly been carried out since 1998 using DAS-ELISA method (Lithuanian State Plant Protection Service). Samples of sugar beet roots with bearded appearance were tested in express lateral flow test (Strube-Dieckmann Spot-check LF^{TM}, Germany) for the fast detection of rhizomania in sugar beet crops in Šakiai and Kaunas regions. Positive results of express lateral flow test were confirmed by other diagnostic methods. One of them was DAS-ELISA. DAS-ELISA – a sensitive immunological method which could detect a very low concentration of virus (1-10 ng /ml) in plant and is suitable to use as a routine method for detection of plants infected with BNYVV. The ELISA test was positive if absorbance of the investigated sample was equal or greater than 3 times the absorbance of the negative (healthy plant) control (Clark and Adams, 1977).

Only in 2004 the first hotbed of the disease was detected in southwest of Lithuania (Šakiai region) and causal agent was identified (Jackeviciene et al., 2005). The second seat of rhizomania was detected in the central part of Lithuania (Kaunas region) in 2005 (Žižytė, et al. 2006; Zizyte and Staniulis, 2007). Third BNYVV isolate was detected in 2007 Panevėžys region (Ėriškių village) (State Plant Protection Service). All three BNYVV isolates were propagated in indicator plants and stored for more detailed studies of the virus. In 2009 rhizomania was detected only in Lithuania sugar beet crops samples in Kaunas region, where the second seat of rhizomania was detected. In Šakiai region, where previously BNYVV was identified, sugar beets were no longer grown. In 2009 sugar beet root samples with suspicious symptoms of rhizomania were also collected in the Ukrainian sugar beet growing areas (Rivnenska, Lvivska, Ternopilska, Ivano-Frankivska, Černivecka, Chmelnycka, Vinnycka and Žytomyrska). 7 samples from Kazakhstan were delivered to us from Kazakhstan's Institute of Agriculture. Beet root samples were analyzed by DAS-ELISA.

So according to the positive DAS-ELISA results, BNYVV was identified in three Lithuanian, ten Ukrainian and in one area of Kazakhstan. These BNYVV isolates were selected for molecular virus analysis. The places of isolates origin and isolates names are entered in Table 2.

Table 2. Selected BNYVV isolates for molecular analysis.

Place of origin	**Isolate name**
Lithuania	
Panevėžys distr., Ėriškių village	Pn
Kaunas distr.	St
Šakiai distr., Tupikų village	T
Ukraine	
Radzyvylivskyj distr., Rivnenska area, Krupec	Ukr2
Brody, Lvivska area	Ukr3
Radechivskyj distr., Lvivska area, Chmilno	Ukr4
Radechivskyj distr., Lvivska area, Babyči	Ukr5
Buzkyj distr., Lvivska area, Ožydiv	Ukr6
Čortkivskyj distr., Ternopilska area, Oryškivci village	Ukr7
Zalicšickyj distr., Ternopilska area, Torske	Ukr8

Gorodenkivskyj distr., Ivano-Frankivska area, Jaseniv-Pilnyj village	Ukr9
Chmelnycka area, Starokostjantyniv	Ukr10
Starokostjantynivskyj distr., Chmelnycka area, Ladygy	Ukr11
Kazakhstan	
Kazakhstan's Institute of Agriculture	Kzt12

Indicator plants in BNYVV analysis. Beet roots samples collected in Lithuania, Ukraine and Kazakhstan sugar beet crops, which according direct DAS-ELISA results were infected with BNYVV, were used for mechanical inoculation of indicator plants.

Host range of BNYVV is restricted mostly by species of the family *Chenopodiaceae* and several species belonging to the *Aizoaceae*, the *Amaranthaceae* and the *Solanaceae*. Usually the virus forms local lesions on mostly inoculated host plant leaves (for example, *Chenopodium quinoa, Tetragonia expansa*). However, virus can infect systemically *Beta macrocarpa*, *Spinacia oleracea* and *Nicotiana benthamiana* (Tamada et al., 1989; Andika et al., 2005).

BNYVV was mechanically inoculated on test and indicator plants recognized as diagnostic species for the BNYVV. Leaves of young indicator plants (in 3-5 leaves stage) were rubbed mechanically with infected sap from fresh or freeze-dried plant material in inoculation buffer. In BNYVV sensitive indicator plants (*C. quinoa, C. amaranticolor*, *T. expansa*) local chlorotic lesions appeared after 5-7 days (Figure 2.) Only in *N. benthamiana* and *S. oleracea* virus caused systemic reactions. Systemic infection of *N. benthamiana* caused slight mottle, curling leaves and stunting of plants, whereas in *S. oleracea* – local clorotic lesions, yellow mosaic flecks and stunting of plants. The obtained results are given in Table 3.

Table 3. Indicator plants reactions to mechanical BNYVV inoculation.

Indicator plant	**Isolate St**		**Isolate T**	
	Symptoms	**Back inoculation**	**Symptoms**	**Back inoculation**
Cucumis sativus L.	-	-	-	-
Nicotiana glutinosa L.	-	-	-	-
N. debneyi Domin	-	-	-	-
N. benthamiana Domin	S, Mo, leaf curling	+	S, Mo, leaf curling	+
N. rustica L.	-	-	-	-
N. tabacum L. cv. 'Samsun'	-	-	-	-
Phaseolus vulgaris L.	-	-	-	-
Chenopodium amaranticolor Coste et Reyn.	LL	+	LL	+
C. quinoa Willd	LL	+	LL	+
Tetragonia expansa Murr	LL	+	LL	+
Atriplex hortensis L.	-	-	-	-
Celosia cristata (L.) Kuntze	-	-	-	-
Spinacia oleracea L.	LL, Mo, S	+	LL, Mo, S	+

LL - local chlorotic lesions, S - systemic infection (stunting of plants), Mo – mosaic flecks, "-" there is no infection, "+" positive back inoculation (produced symptoms are the same as the primary infected indicator plants).

The obtained results correspond to BNYVV host plant range denoted in literature and symptoms caused of this virus (Tamada and Baba, 1973; Tamada et al., 1989; Andika et al., 2005).

C. quinoa, C. amaranticolor, *T. expansa* – locally infected indicator plants were selected for BNYVV propagation and storage (Fig. 2.).

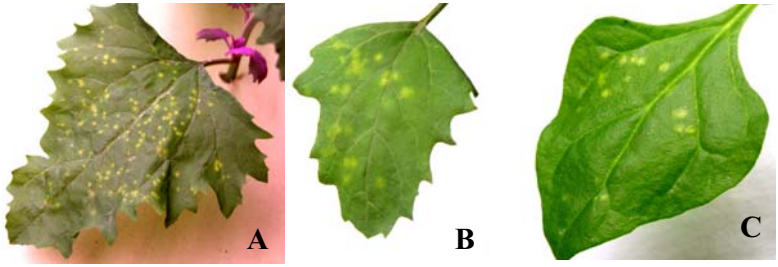

Fig. 2. BNYVV mechanical inoculation: local chlorotic lesions on indicator plant leaf (**A.** *Chenopodium amaranticolor* **B.** *C. quinoa* **C.** *Tetragonia expansa*).

Infected plant material with obvious symptoms was collected and tested by direct DAS-ELISA, electron microscopy (EM), weighed, labelled and stored at minus temperature. In particular, *C. quinoa* indicator plant was chosen for virus propagation because it gives the best yield of BNYVV (Bouzoubaa, 1998), required for virus purification.

Morphology of rhizomania agent. Electron microscopy method was used for viral particles from infected plants (both from sugar beet roots and mechanically inoculated indicator plants) detection, identification and morphological features analysis. For virus particles detection also was used immunosorbent electron microscopy (IEM), developed by Derrick (1973), in which viral particles were trapped from a suspension by specific antibodies which were coated on the grid. In IEM viral particles become selectively attached to the grid, whereas the unbound host plant material is easily removed, and the viral particles are concentrated. Test samples were stained with negative dye (2 % UA) and analyzed by JEOL JEM-100S transmission EM. Figures 3 and 4 represent EM and IEM preparations of BNYVV particles derived from infected plants.

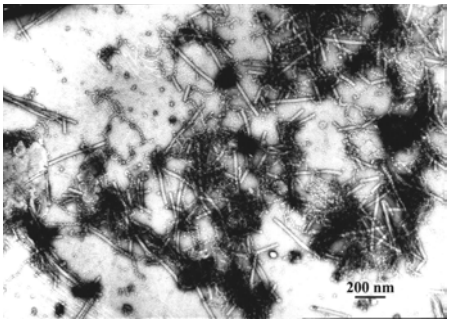

Fig. 3. EM (magnification 20000 ×): BNYVV viral particles, from mechanical inoculated *C. quinoa* extract.

EM and IEM of investigated plant preparations with typical symptoms of BNYVV showed varying length, about 20 nm in diameter, straight rod-shaped BNYVV particles. This corresponds to the data provided in the literature (Richards and Tamada, 1992; Fauquet et al., 2005).

EM was also used in BNYVV clarification, concentration stages and in analysis of the purified virus preparations (Žižytė and Staniulis, 2007b).

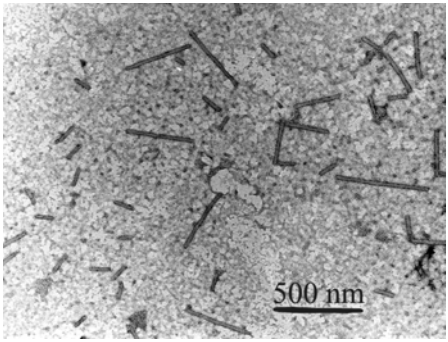

Fig. 4. IEM (magnification 20000 ×): about 20 nm in diameter BNYVV characteristic viral particles, from sugar beet rootlets extract.

Detection of BNYVV using PCR methods. For BNYVV identification one or two step conventional RT-PCR, IC-RT-PCR and nPCR were used. Conventional RT-PCR was carried out using specific primers of the read-through region of the coat protein of BNYVV which amplify a 500 bp fragment (Henry et al., 1995; OEPP/EPPO, 2004). For amplification of this fragment virus RNA, extracted from mechanically inoculated indicator plants with detected Lithuanian BNYVV isolates, was used. Figure 5. A illustrates products produced in the RT-PCR.

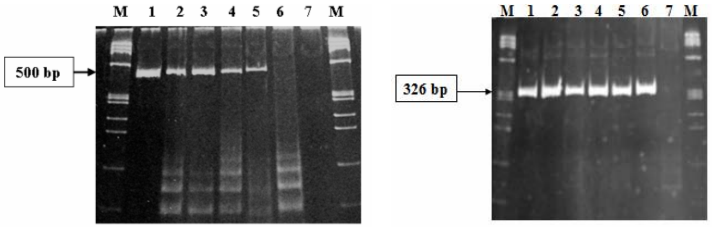

Fig. 5. PCR products in 5 % bisacrylamide gel: A. **RT-PCR:** M – marker Φ×174 DNA/BsuRI(Hae III); 1–4 – samples of St isolate detected in Lithuania; 5 – K+; 6 – K–; 7 – Kw. **B. nPCR:** M – marker Φ×174 DNA/BsuRI(Hae III); 1 – K+; 2–6 – samples of St isolate detected in Lithuania; 7 – K–.

In order to increase BNYVV detection level, nPCR method was used. It improves the sensitivity of virus detection by 1000-fold compared with the conventional RT-PCR (Morris et al., 2001). nPCR was performed with internal primers, which annealed to the amplicon (500 bp), produced from standard PCR, and specifically amplified a 326 bp product (Fig. 5. B).

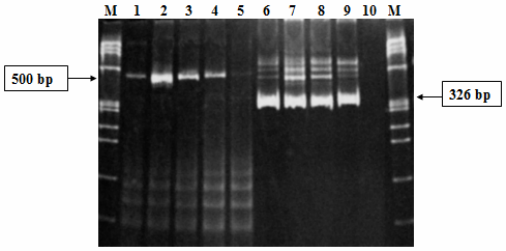

Fig. 6. RT-PCR products in 5 % bisacrylamide gel: M – marker Φ×174 DNA/BsuRI(Hae III); 1–3 – IC-RT-PCR products of St isolate detected in Lithuania; 4 – K+; 5 – K–; 6–8 – nIC-RT-PCR products; 9 – K+; 10 – Kw.

For BNYVV identification was also used IC-RT-PCR. In this method the same primers were used as in conventional RT-PCR. Uniqueness of the method is that pathogen initially was caught with antibodies against BNYVV as in DAS-ELISA

method, and then RT-PCR was performed under the same conditions as in conventional RT-PCR. 500 bp products (Fig. 6. 1-5 lines) produced in this reaction were also used to perform nPCR (Fig. 6. 6-10 lines).

nPCR method is recommended to use for improvement of conventional RT-PCR sensitivity and specificity, necessary for early detection of viral infection and assessment of sugar beet resistance or tolerance to BNYVV (Morris et al., 2001).

Determination of BNYVV types. It is known, that BNYVV has three main (A, B and P) types, which can not be distinguished serologically. But using restriction fragment length polymorphism analysis, multiple RT-PCR (mRT-PCR) and comparing the virus coat protein sequences it is possible to determine the types of BNYVV isolates.

RFLP analysis was based on the different restriction endonucleases cleavage of the certain genome region. For RFLP analysis BNYVV RNA 2 genomic region (2133-3293 nt) was chosen which encodes an 42 kDa protein. Amplified by RT-PCR, this virus fragment (1160 bp), may have a specific recognition sequence for restriction endonucleases (*Taq*I, *Hinc*II, *Acc*I and *BstU*I) which determine BNYVV A or B type (Kruse et al., 1994).

RFLP typing method was used for isolates (St, T and Pn) detected in Lithuania. In the case of BNYVV A type, 1160 bp fragment should not be recognized by *Taq*I and *Hinc*II, only *Acc*I and *BstU*I should recognize the fragment. In B type case, conversely, *Taq*I and *Hinc*II recognize 1160 bp fragment, and *Acc*I and *BstU*I – do not. Figure 7. illustrates restriction profiles of the 1160 bp fragment with *Taq*I (Fig. 7. A), *Hinc*II (Fig. 7. B), *Acc*I (Fig. 7. C) and *BstU*I (Fig. 7. D) restriction endonucleases.

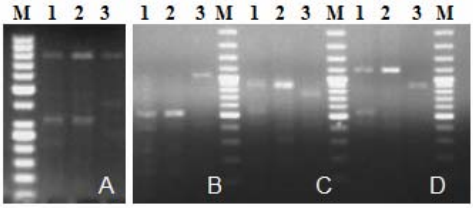

Fig. 7. RFLP typing of isolates detected in Lithuania.
A: restriction profile of *Taq*I: M – GeneRuler 50 bp DNA Ladder; 1 – St; 2 – T; 3 – Pn; **B: restriction profile of *Hinc*II:** M – GeneRuler 100 bp Plus Ladder; 1 – St; 2 – T; 3 – Pn; **C: restriction profile of *Acc*I:** M – GeneRuler 100 bp Plus Ladder; 1 – St; 2 – T; 3 – Pn; **D: restriction profile of *BstU*I:** M – GeneRuler 100 bp Plus Ladder; 1 – St; 2 – T; 3 – Pn.

According to the obtained restriction profiles, *Taq*I and *Hinc*II restriction endonucleases cleaved 1160 bp fragment of St and T isolates detected in Lithuania (Fig. 7. A and B: 1 and 2 lines), and *Acc*I and *BstU*I – did not (Fig. 7. C and D: 1 and 2 lines), so these isolates belong to B type. In the case of Pn isolate opposite results were obtained – *Taq*I and *Hinc*II restriction endonucleases did not cleave the 1160 bp fragment (Fig. 7. A and B: 3 line), *Acc*I and *BstU*I – cleaved (Fig. 7. C and D: 3 line), so Pn isolate belongs to A type.

mRT-PCR for BNYVV type detection was performed in two stages. The first RT-PCR stage was carried out using specific primers for triple gene block (TGB) fragment (722 bp) amplification. In the second stage nPCR was carried out using amplified TGB gene fragment, and two pairs of A and B type specific primers (Ratti, et al., 2005). Amplified 324 bp product would show A type isolates, whereas 178 bp product – B type isolates. Figure 8. illustrates amplification of TGB (Fig. 8. A) and A/B type products (Fig. 8. B).

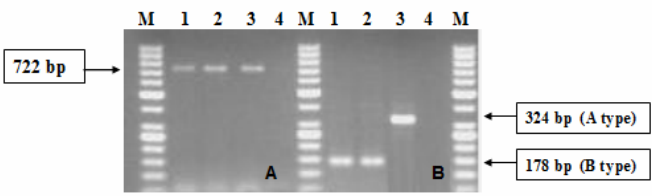

Fig. 8. PCR products of isolates detected in Lithuania in 2 % agarose gel. A: TGB products: M – GeneRuler 50 bp DNA Ladder; 1 – St; 2 – T; 3 – Pn; 4 – K–; **B: A/B type products:** M – GeneRuler 50 bp DNA Ladder; 1 – St; 2 – T; 3 – Pn; 4 – K–.

In figure 8. B 1st and 2nd lines (St and T isolates) 178 bp size product was amplified, which is characteristic of B type. In 3rd line (Pn isolate) 324 bp size product was obtained, specific for A type. No amplification product was observed in 4th lines (healthy *C. quinoa* plant) of figure 8. These results confirmed the RFLP analysis.

mRT-PCR was also used for BNYVV types detection of Ukrainian isolates and isolate from Kazakhstan. Fig. 9. illustrates the second stage of mRT-PCR (nPCR) – A/B type amplification products of BNYVV isolates detected in Ukraine and Kazakhstan.

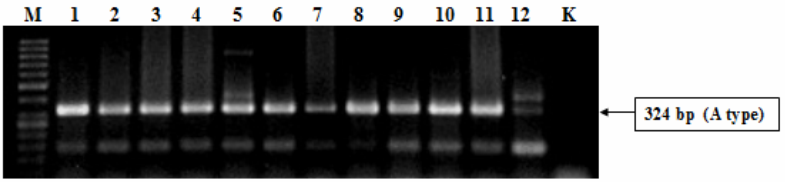

Fig. 9. A/B amplification products of isolates detected in Ukraine and Kazakhstan in 2 % agarose gel: M – GeneRuler 50 bp DNA Ladder; 1–2 – ukr2; 3 – ukr3; 4 – ukr4; 5 – ukr5; 6 – ukr6; 7 – ukr7; 8 – ukr8; 9 – ukr9; 10 – ukr10; 11 – ukr11; 12 – kzt12; K – water control.

In figure 9. in all lines 324 bp size products were obtained, so all isolates detected in Ukraine and Kazakhstan by the mRT-PCR method could be assigned to BNYVV A type.

Phylogenetic analysis of BNYVV isolates. **Phylogenetic study of CP**. To get more information about diversity of detected BNYVV isolates, and to confirm BNYVV types, specific primers (Schirmer et al., 2005) have been selected, which amplify CP gene (567 bp). RNA 2 5' proximal ORF encodes a 21-kDa CP. This protein was chosen for analysis, as the nature of the amino acid residues 62, 103 and 172 permitted us to distinguish between BNYVV A type (T_{62}, S_{103}, L_{172}) and B type (S_{62}, N_{103}, F_{172}) isolates (Kruse et al., 1994; Koenig et al., 1995; Miyanishi et al., 1999). In addition, according to the specific amino acid of CP, A type isolates could be distinguished from P type. It is known that A and P types are more related than B type (Koenig and Lennefors, 2000, Meunier et al., 2005; Ratti et al., 2005; Schirmer et al., 2005). P21 sequences of BNYVV A type isolates have more similarity with P type than B type isolates (97.9 compared to 95.2 %) (Koenig and Lennefors, 2000). A Type isolates with RNA 5 and CP specific amino acid residues R_{17} and I_{102} could be assigned to P type isolates (Koenig et al., 1995; Miyanishi et al., 1999).

For amplification of CP gene the virus RNA, extracted from infected sugar beet roots and indicator plants mechanically inoculated with BNYVV (Lithuanian, Ukrainian isolates and isolate from Kazakhstan), was used. Figure 10. illustrates the RT-PCR products of p21 gene. In all cases specific BNYVV CP (567 bp) product was obtained. This is in coincidence with the direct DAS-ELISA results. Obtained CP products were purified and sequenced.

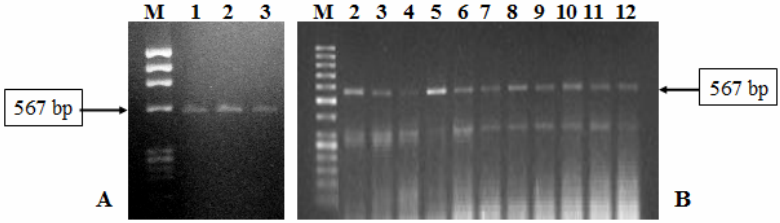

Fig. 10. Amplification products of CP gene in 2 % agarose gel: A (isolates detected in Lithuania): M – marker ΦX174 DNA/BsuRI(Hae III); 1 – St; 2 – T; 3 – Pn; **B (isolates detected in Ukraine and Kazakhstan):** M – GeneRuler 50 bp DNA Ladder; 2 – ukr2; 3 – ukr3; 4 – ukr4; 5 – ukr5; 6 – ukr6; 7 – ukr7; 8 – ukr8; 9 – ukr9; 10 – ukr10; 11 – ukr11; 12 – kzt12.

For eight isolates (Lithuanian St, T, Pn; Ukrainian ukr2, ukr9, ukr10, ukr11 and kzt12 isolate from Kazakhstan) CP nucleotide sequence was determined. For six isolates (St, T, ukr2, ukr9, ukr10, ukr11) CP gene sequence (567 bp) was fully determined, and for remaining two isolates (Pn and kzt12) – only partially. CP protein amino acid sequence (188 aa) was derived from the obtained nt sequence. Obtained sequences were aligned with each other and with other sequences from the gene bank database (Table 4.).

Table 4. Accession numbers of BNYVV isolates RNA 2 from gene bank database.

Country	Isolate	Accession number of RNA 2	BNYVV CP type	RNA 5 P26	Reference
Austria					
	A2	AY696072	A	-	Schirmer et al., 2005
	A4	AY696075	A	-	Schirmer et al., 2005
Belgium					
	Beauvechain	AJ634732	B	-	Meunier et al., 2005
	Mazy	AJ634736	B	-	Meunier et al., 2005
	Rutten	AJ634737	B	-	Meunier et al., 2005
	St Germain	AJ634738	B	-	Meunier et al., 2005
	Zetrud	AJ634741	B	-	Meunier et al., 2005
	B3	AY696079	B	-	Schirmer et al., 2005
	Illko.01	AY696099	B	-	Schirmer et al., 2005
	Illko.96	AY696102	B	-	Schirmer et al., 2005
	Beclers	AJ634733	A	-	Meunier et al., 2005
	B1-(2)	AY734492	A	-	Schirmer et al., 2005
Iran					
	IR-GR	AM779752	A	P	unpublished
	Iran Fars	AY277887	A	NA	Schirmer et al., 2005
Spain					
	S3	AY696110	A	-	Schirmer et al., 2005

	S4	AY696111	A	-	Schirmer et al., 2005
	S5	AY696112	A	-	Schirmer et al., 2005
	S10	AY696114	A	-	Schirmer et al., 2005
Japan					
	SH1	AB018621	A	J	Miyanishi et al., 1999
	S42	AB018622	A	J	Miyanishi et al., 1999
	S12	AB018623	B	J	Miyanishi et al., 1999
	S44	AB018624	B	J	Miyanishi et al., 1999
	D104	AB018620	A	J	Miyanishi et al., 1999
USA					
	B2	AY696078	A	-	Schirmer et al., 2005
	A3	AY696074	A	-	Schirmer et al., 2005
	UF79	AJ810285	A	-	Lennefors et al., 2005
	1EU	AY696070	A	-	Schirmer et al., 2005
	412	AY696118	A	-	Schirmer et al., 2005
	S-89	AY771347	A	NA	unpublished
Kazakhstan					
	Kas3	AF197556	A	P	Koenig and Lennefors, 2000
China					
	CY3	AB018625	B	J	Miyanishi et al., 1999
	CH2	AB018626	B	J	Miyanishi et al., 1999
	NM	S71490	B	-	Yao et al t., 1993
	Yao 2	U25668	B	-	Yao et al., 1993
	Har2	EF473089	A	J	Li et al., 2008
	Hoh2	EF473092	B	J	Li et al., 2008
	Wu2	EF473095	A	J	Li et al., 2008
	Chan1	EF473099	B	J	Li et al., 2008
Poland					
	ZAcp	EU785964	A	NA	unpublished
	JAcp	EU785963	A	NA	unpublished
Netherlands					
	NL3	AY696105	A	-	Schirmer et al., 2005
France					
	FC	AB018627	A	P	Miyanishi et al., 1999
	FP	AB018628	A	P	Miyanishi et al., 1999
	EP32A	AY696085	B	-	Schirmer et al., 2005
	EP38	AY696087	B	-	Schirmer et al., 2005
	C18-1	AY734493	B	-	Schirmer et al., 2005
	FP92	AJ810283	A	P	Lennefors et al., 2005
	F75	AF197547	A	P	Koenig and Lennefors, 2000
	EP42	AY696089	A	+	Schirmer et al., 2005
	EP32B	AY696086	A	-	Schirmer et al., 2005
	Fb2	AY696094	B	-	Schirmer et al., 2005
	H4	AY696097	A	P	Schirmer et al., 2005
	F13	X04197	B	-	Bouzoubaa et al., 1986
Serbia and Montenegro					
	A	AY920465	A	NA	unpublished
Germany					
	G2	AY696096	B	-	Schirmer et al., 2005
	GT85	AJ810284	B	-	Lennefors et al., 2005
	OW1	EU864118	A	+	Koenig et al., 2008

A – A type CP; B – B type CP; P – P type P26 protein; J – Asian type P26 protein, NA – not available, "-" absence of RNA 5, "+" presence of RNA 5.

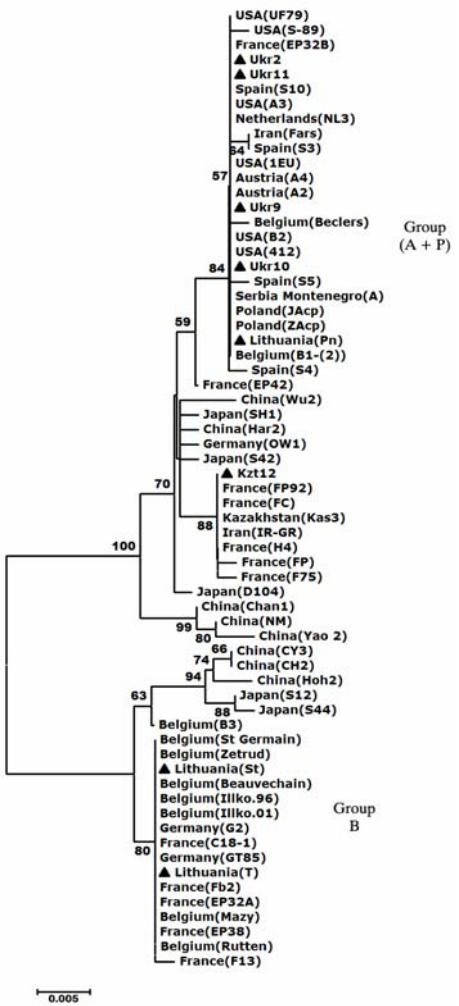

Fig. 11. Phylogenetic tree of BNYVV RNA 2 encoded CP gene sequences, computed by MEGA 4.1 using the neighbor-joining method. Names refer to the geographical origin and name of the isolate. (For sequences accession numbers, see Table 4.). In this study determined sequences of BNYVV isolates are marked with ▲. Scale of tree shows phylogenetic similarity index (bar refers to 0.005 nt changes per site). Numbers on branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 50 are shown.

The identity of CP gene nt and aa sequences ranged from 95 to 100 %. Analyzed isolates did not reveal any nt differences in conserved gene region (*data not shown*). However, between two isolates detected in Lithuania (St and T) and between four isolates detected in Ukraine (ukr2, ukr9, ukr10, ukr11) with one isolate detected in Lithuania (Pn) nucleotide sequences were identical.

Only the most distinct nt sequences from gene data bank were included in the phylogenetic tree presented in figure 11. Phylogenetic analysis of CP gene divided all BNYVV isolates in two main groups. One group contained the A and P type isolates (Group A + P), and included isolates ukr2, ukr9, ukr10, ukr11, kzt12 and Pn investigated in this study. Another group consisted of B type isolates (Group B) and included St and T isolates (Fig. 11.).

Based on the previous studies (Kruse et al., 1994; Koenig et al., 1995; Miyanishi et al., 1999), A + P group and B group could be discriminated by CP amino acid changes at three positions 62 (T to S), 103 (S to N) and 172 (L to F). European isolates with RNA 5 are designated as P type (Koenig et al., 1995), but otherwise are very closely related to A type (Miyanishi et al., 1999).

Significant CP amino acids changes of BNYVV isolates investigated in this study are given in Table 5.

Table 5. Amino acids changes of investigated BNYVV isolates

Isolate	Country	Amino acids residues in P21					BNYVV type
		17	**62**	102	**103**	**172**	
St	Lithuania	K	**S**	V	**N**	**F**	B
T	Lithuania	K	**S**	V	**N**	**F**	B
Pn	Lithuania	K	**T**	V	**S**	**-**	A
Ukr2	Ukraine	K	**T**	V	**S**	**L**	A
Ukr9	Ukraine	K	**T**	V	**S**	**L**	A
Ukr10	Ukraine	K	**T**	V	**S**	**L**	A
Ukr11	Ukraine	K	**T**	V	**S**	**L**	A
Kzt12	Kazakhstan	R	**T**	I	**S**	**-**	A(P*)

Amino acid changes, which are used to distinguish BNYVV type (A or B) noted in bold letters, "-" unidentified. * P type is named by Koenig et al., 1995, and Miyanishi et al., 1999.

Group B of phylogenetic CP gene tree included all B type isolates from Europe and Asia. BNYVV isolates detected in Europe did not have RNA 5, and isolates from Asia – had. This group included isolates St and T (Lithuania, this study).

Group A + P is very diverse. CP gene sequence of isolate kzt12 (this study) was identical to Kas3 (Kazakhstan), FC, FP92, H4 (France) and IR-GR (Iran) sequences. These six isolates, and isolates FP and F75 (France) are A type isolates with RNA 5 and contained P type specific CP aa residues (R_{17} and I_{102}) (Koenig et al., 1995; Miyanishi et al., 1999), so these isolates could be assigned to P type isolates.

The group A + P also included isolates from Japan (S42, SH1 and D104), China (Har2, Wu2 and Chan1) and Germany (OW1) containing RNA 5 (Miyanishi et al., 1999; Koenig et al., 2008, Li et al., 2008). All Japanese and Chinese isolates in RNA 5 P26 sequence had two deletions at position 77 and 227-229, whereas the European isolates – had none. Therefore, isolates from East Asia belong to J type and European isolates – to P type (Koenig and Lennefors, 2000; Schirmer et al., 2005; Li et al., 2008). German isolate (OW1) RNA 5 sequence is closely related to the East Asian isolates and differs from Europe isolates with RNA 5. This is the first isolate with RNA 5 identified in Germany (Koenig et al., 2008).

All other isolates remaining in this group belong to BNYVV A type with no RNA 5, among which we find isolates ukr2, ukr9, ukr10, ukr11 and Pn investigated in this study (Fig. 11.).

Interestingly, three Chinese (NM Yao2 and Chan1) B type isolates (Yao et al., 1993, Li et al., 2008), were closely related to this A + P group although they were clearly distinct from other isolates according to phylogenetic analysis of CP nt sequences.

Slightly different phylogenetic tree is constructed from multiple BNYVV isolates CP amino acids sequences alignment (Fig. 12.).

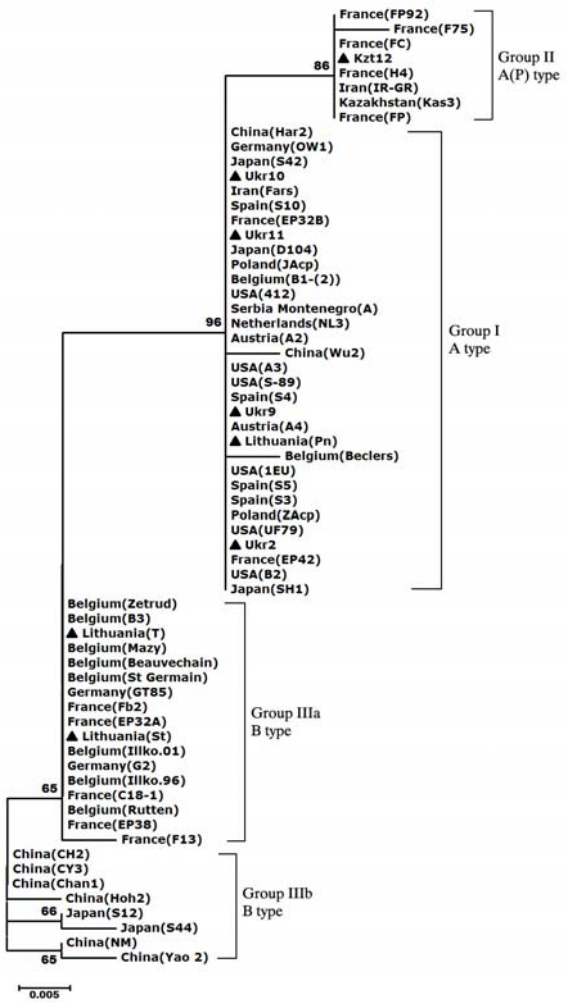

Fig. 12. Phylogenetic tree of BNYVV RNA 2 encoded CP protein sequences, computed by MEGA 4.1 using the neighbor-joining method. Names refer to the geographical origin and name of the isolate. (For sequences accession numbers, see Table 4.). In this study determined sequences of BNYVV isolates are marked with ▲. Scale of tree shows phylogenetic similarity index (bar size refers to 0.005 amino acid changes per site). Numbers on branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 50 are shown.

Phylogenetic tree displayed three main groups: two of them were associated with A type (Groups I and II) and one (Group III) with B type isolates. This tree more precise grouped BNYVV isolates than phylogenetic tree of CP nt sequences.

CP sequences of BNYVV isolates within Group I (Fig. 12.) shared 100 % identity, regardless of their geographical origin, except Belgian (Beclers) (Meunier et al., 2005) and Chinese (Wu2) (Li et al., 2008) isolates which differed from others A type isolates due to S_3I and $F_{168}L$ substitution, respectively. Within this group are isolates ukr2, ukr9, ukr10, ukr11 and Pn investigated in this study. Some isolates of this group had RNA 5 (all East Asian isolates, German OW1 and French EP42) (Table 4.).

Similarly, within Group II all BNYVV CP sequences were identical except French (F75) isolate, which differed from others due to $L_{41}S$ substitution. This group included isolate kzt12 investigated in this study. All members of this group contained RNA 5. There were no Chinese and Japanese isolates, only Asian isolates from Kazakhstan (Koenig and Lennefors, 2000) and Iran. This group could be called P type, because all isolates are A type with RNA 5 and contained P type specific CP aa residues (R_{17} and I_{102}) (Koenig et al., 1995; Miyanishi et al., 1999).

Group III was more diverse, it included all European and Asian B type isolates. Within Group IIIa European isolates were without RNA 5. This group included isolates St and T from Lithuania (this study). Meanwhile, within Group IIIb Asian isolates contained RNA 5 (except Chinese Yao2 and NM). Isolates NM, Yao2 and Chan1 belonged to this group in contrast to phylogenitic analysis of CP nt sequences (Fig. 11.). Finally, French isolate F13 differed from others isolates in group IIIa and IIIb due to $N_{64}S$ substitution.

Phylogenetic analysis of CP protein sequences confirmed and itemized types of investigated BNYVV isolates. According to CP protein sequence analysis Lithuanian isolates St and T belonged to B type, Lithuanian Pn and all Ukrainian isolates (ukr2, ukr9, ukr10, ukr11) – to A type and isolate from Kazakhstan (kzt12) – to P type.

Phylogenetic study of BNYVV p25. BNYVV P25 protein is responsible for the spread of the virus in sugar beet roots and the production of the typical rhizomania symptoms (Tamada et al., 1989; Koenig et al., 1991; Tamada et al., 1999). Therefore, it is considered that BNYVV RNA 3 is a pathogenicity determinant, and P25 is responsible for the symptoms of rhizomania in sugar beets (Jupin et al., 1991; Chiba et al., 2008).

High P25 protein sequence variability is detected particularly within four amino acids tetrad (residues 67-70), especially in A type BNYVV (Schirmer et al., 2005; Yilmaz et al., 2007; Chiba et al., 2008, Koenig et al., 2008; Li et al., 2008). P25 protein consists of 219 amino acids. Significant amino acid tetrad is located downstream of the nuclear localization signal motif (Vetter et al., 2004) and upstream of the zinc finger motif (Jupin et al., 1992). The variations of tetrads may have a strong influence on P25 oligomerization and on BNYVV pathogenicity in *Tetragonia expansa* (Klein et al., 2007) and partially resistant sugar beet varieties (Liu and Lewellen, 2007; Acosta-Leal et al., 2008, Chiba et al., 2008).

For BNYVV p25 gene identification specific primers were selected, which amplified 719 bp product (Schirmer et al., 2005). Three Lithuanian and one Ukrainian isolates were used for this gene analysis. In all cases specific BNYVV p25 (719 bp) product was obtained (Fig. 13.).

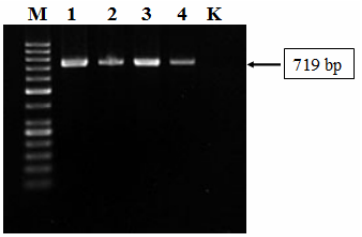

Fig. 13. Amplification products of p25 gene in 2 % agarose gel: M – GeneRuler 50 bp DNA Ladder; 1 – St; 2 – T; 3 – P; 4 – Ukr2; K – water control.

Obtained specific products (719 bp) were purified, sequenced and used for phylogenetic analysis. For two isolates (St and T) p25 gene sequence was fully determined, and for others (Pt and ukr2) – only partially. P25 protein amino acid sequences (219 aa.) were derived from obtained nt sequences. P25 protein sequences showed that investigated St and T isolates contained AYHR tetrad (in 67-70 position), Pn – AHHG and ukr2 – AYHG tetrad.

According to the previous CP analysis, Lithuanian Pn and Ukrainian isolates were assigned to BNYVV A type and Lithuanian St and T isolates – to B type. Multiple P25 protein amino acid sequence alignment showed that sequences of Lithuanian B type isolates resemble sequences of Belgian and German B type isolates. Sequences of A type isolates (ukr2 and Pn) resembles sequences of Belgian, French and Swiss BNYVV A type isolates. Accession number of BNYVV RNA 3 sequences used for alignment noted in Table 6.

Phylogenetic tree of P25 protein displayed three major groups, two of which were associated with A type (group P25-I and P25-II) and one (group P25-III) – with B type isolates (Fig. 14.). This corresponded to Schirmer et al. (2005) given phylogenetic analysis of P25 protein of BNYVV collected worldwide.

According to phylogenetic tree analysis of P25 protein, two B type isolates St and T (this study) were assigned to P25-III group together with the B type isolates containing AYHR tetrad from Belgium, Germany, France and the Czech Republic (Fig. 14.). This is consistent with Schirmer et al. (2005) estimated correlation of European isolates between the CP, P25 and RNA 5, in which A type CP isolates have multiple tetrad motives of P25 and almost do not contain RNA 5 component; B type CP isolates have a uniform tetrad motif AYHR and do not contain RNA 5; P type CP isolates have SYHG tetrad and contain RNA 5 (P type P26) (Schirmer et al., 2005).

Table 6. Accession numbers of BNYVV isolates RNA 3 from gene bank database.

Country	Isolate	Accession number of RNA3	Amino acid tetrad (in 67-70 position)	BNYVV CP type	Reference
Austria					
	A2	AY696123	AFHG	A	Schirmer et al., 2005
	A4	AY696126	AFHG	A	Schirmer et al., 2005
Belgium					
	B3	AY696130	AYHR	B	Schirmer et al., 2005
	Illko.01	AY696158	AYHR	B	Schirmer et al., 2005
	Illko.96	AY696161	AYHR	B	Schirmer et al., 2005
	B1-(2)	AY734498	AHHG	A	Schirmer et al., 2005
Czech Republic					
	Cz4	DQ462121	AYHR	B	Ward et al., 2007
Iran					
	BNQ1	AY703454	AHHG	A	unpublished
Spain					
	S3	AY696169	VCHG	A	Schirmer et al., 2005

	S4	AY696170	ACHG	A	Schirmer et al., 2005
	S5	AY696171	VCHG	A	Schirmer et al., 2005
	S10	AY696173	AFHG	A	Schirmer et al., 2005
Italia					
	I12	AF197551	ALHG	A	Koenig ir Lennefors, 2000
Japan					
	S	D84412	AYRV	A	Saito et al., 1996
	O11	AB207805	AFHG	A	Chiba et al., 2008
	Japon	AY696163	AYHG	A	Schirmer et al., 2005
	S113	AB207806	AYRV	A	Chiba et al., 2008
USA					
	B2	AY696078	VLHG	A	Schirmer et al., 2005
	A3	AY696125	VLHG	A	Schirmer et al., 2005
	1EU	AY696119	ALHG	A	Schirmer et al., 2005
	412	AY696121	ACHG	A	Schirmer et al., 2005
	IV-8	DQ415513	SYHG	A	unpublished
United Kingdom					
	FF	DQ440513	TYHG	A	Ward et al., 2007
	MH	DQ440512	SYHG	A(P)	Ward et al., 2007
Kazakhstan					
	Kas2	AF197553	ALHG	A(P)	Koenig ir Lennefors, 2000
	Kas3	DQ462126	SYHG	A(P)	Ward ir kt., 2007
China					
	NM	AJ239200	AYHG	B	Li ir kt., 1998
	Har2	EF473100	AFHG	A	Li ir kt., 2008
	Har4	EF473101	AFHG	A	Li ir kt., 2008
	Hoh2	EF473103	AYHG	B	Li ir kt., 2008
	Wu2	EF473108	AHHR	A	Li ir kt., 2008
	Chan1	EF473110	AFHR	B	Li ir kt., 2008
Netherlands					
	NL3	AY696164	AFHR	A	Schirmer et al., 2005
France					
	EP32A	AY696136	AYHR	B	Schirmer et al., 2005
	EP38	AY696140	AYHR	B	Schirmer et al., 2005
	EP42	AY696143	SYHG	A(P)	Schirmer et al., 2005
	EP32B	AY696137	AFHR	A	Schirmer et al., 2005
	H4	AY696156	SYHG	A(P)	Schirmer et al., 2005
	C18-1	AY734499	AHHG	A	Schirmer et al., 2005
	Pith.	AY734505	SYHG	A(P)	Schirmer et al., 2005
	EP24	AY696134	SYHG	A(P)	Schirmer et al., 2005
Sweden					
	S8	EU330454	AHHG	A	Koenig et al., 2008
Switzerland					
	Ch40	EU770694	AFHG	A	Koenig et al., 2008
	Ch6	DQ462118	AHHG	A	Ward et al., 2007
Turkey					
	T1	AY772230	AHHG	A	Yilmaz et al., 2007
	T11	AY772234	ACHG	A	Yilmaz et al., 2007
Germany					
	G2	AY696155	AYHR	B	Schirmer et al., 2005
	OW01	EU770693	AFHG	A	Koenig et al., 2008
	D15	DQ462122	AHHR	B	Ward et al., 2007

A – A type; B – B type; (A)P – P type isolate.

* European isolates with RNA 5 are designated as P type (Koenig ir kt., 1995), but otherwise very closely related to A type (Miyanishi ir kt., 1999).

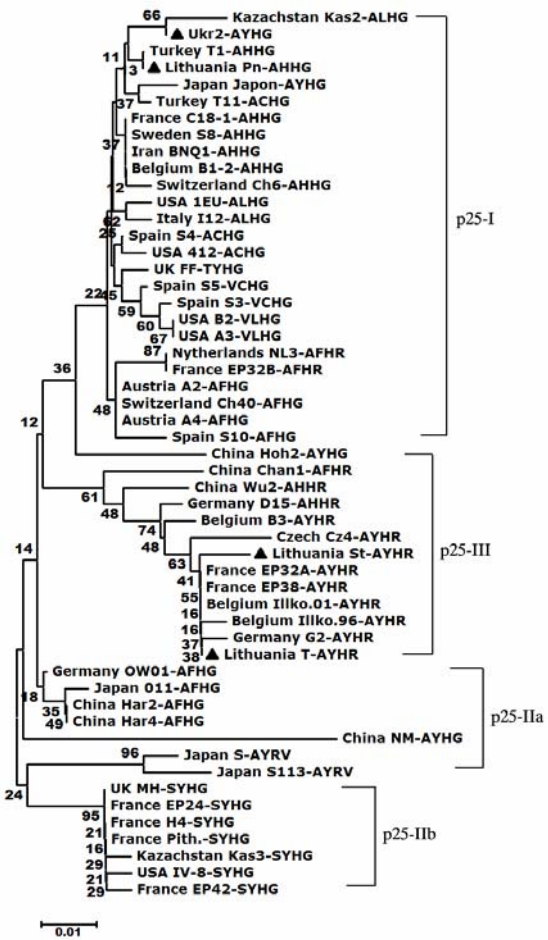

Fig. 14. Phylogenetic tree of BNYVV RNA 3 encoded P25 protein sequences, computed by MEGA 4.1 using the neighbor-joining method. Names refer to the geographical origin and name of the isolate. (For sequences accession numbers, see Table 6.). In this study determined sequences of BNYVV isolates are marked with ▲. The tetrad of P25 protein is separated by a hyphen. Scale of tree shows phylogenetic similarity index (bar size refers to 0.01 amino acid changes per site). Numbers on branches represent the bootstrap values out of 1000 replicates.

It is interesting that German isolate D15 with AHHR tetrad and Chinese isolates, one of which (Wu2) belongs to A type with AHHR tetrad, and the other – to B type (Chan1) with AFHR tetrad, are assigned to this P25-III group. Chinese isolates does not have similar relation to Schirmer et al. (2005) estimated correlation for European isolates. Chinese and Japanese isolates containing RNA 5 (J type P26) are associated with A and B type CP isolates and variable tetrad motif of P25. Thus, East Asian isolates may share a common origin (Miyanishi et al., 1999; Li et al., 2008).

Whereas, German isolate D15 is a "deviant" B type isolate coding P25 with AHHR tetrad. This sequence contained silent mutations at nt positions 306 (g→a), 354 (c→t) and 642 (t→c) nt. BNYVV B type isolates with AHHR was also found in Switzerland (Koenig et al., 2008).

A type isolates Pn and ukr2 (this study) were assigned to P25-group I (Fig. 14.). P25-I group contained mainly A type isolates (from Austria, Switzerland, Spain, Netherlands, France, Italy, Kazakhstan, Belgium, Iran, United Kingdom, Sweden, Turkey, United States and Japan) and one B type Chinese isolate (Hoh2). Within this group nine of thirteen different tetrad motifs were found. Some of isolates contained RNA 5 (Kas2 and Japon).

In this study investigated isolates were not assigned to P25-II group, which was separated into two subgroups: P25-IIa and P25-IIb.

P25-IIb group contained only A type isolates that expressed P25 with the unique SYHG tetrad and had RNA 5. This group included almost all European P type isolates (from France, United Kingdom), P type isolate (Kas3) from Kazakhstan and American A type isolate. Group P25-IIa contained A type Japanese isolates mostly with AYRV tetrad, except of one isolate with AFHG tetrad (O11). Most of isolates contained RNA 5 (except Japanese isolates O11 and S113, and Chinese NM). German isolate OW01 with AFHG tetrad also belonged to this group. P25 sequence of this isolate was more similar to the P25 from East Asia rather than European BNYVV isolates (Koenig et al., 2008).

It is interesting that Chinese isolates Hoh2 and NM were assigned to P25-I and P25-IIa groups of A type isolates, although these isolates contained B type CP. Also A type isolate Wu2 assigned to P25-III group consisted of B type isolates. This indicates that these isolates represent a natural re-assortment resulting from exchanges of RNA 3 between the isolates with A and B type CP (Li et al., 2008).

P25 sequence variation may influence the degree of pathogenicity of BNYVV isolates. RNA 3 variability may overcome resistance in partially resistant sugar beet varieties (Schirmer et al., 2005; Klein et al., 2007).

According to phylogenetic analysis of P25 protein Lithuanian BNYVV St and T isolates were assigned to B type CP isolates with AYHR tetrad, Lithuanian Pn – to A type CP isolates with AHHG and Ukrainian ukr2 – to A type CP isolates with AYHG tetrad. This is consistent with Schirmer et al. (2005) estimated correlation of BNYVV isolates between CP, P25 and RNA 5.

Detection of others beet viruses, related with rhizomania. Three viruses - BNYVV, BSBV and BVQ are transmitted by the same vector *P. beta*e Keskin. Although influence of these two (BSBV and BVQ) pomoviruses in rhizomania infection remains at the level of debate, often in rhizomania infected crops they are found together with BNYVV in sugar beet roots (Prillwitz and Schlosser, 1992; Meunier et al., 2003). It was interesting to examine whether these viruses would be detected together with investigated BNYVV isolates in this study.

BNYVV and BSBV were detected by direct DAS-ELISA and direct TAS-ELISA, respectively. BVQ detection was relied on multiple RT-PCR, allowing simultaneously identify BNYVV, BSBV and BVQ. In Lithuanian isolates (ST, T, Pn) mixture of both BNYVV and BSBV was not detected (*data not shown*). Meanwhile, in some Ukrainian isolates and isolate from Kazakhstan, these viruses were found together. BSBV detection was carried out by both ELISA and mRT-PCR. For BSBV detection by TAS-ELISA

only isolates in which have previously been identified BNYVV were used. Obtained results of ELISA are shown in Table 7.

According to TAS-ELISA results BSBV was detected in ukr2, ukr4, ukr9, ukr10, ukr11 and kzt12 isolates (Table 7.).

Table 7. ELISA results of investigated BNYVV and BSBV mix infection in Ukrainian isolates and isolate from Kazakhstan.

Isolates	Virus infection	
	BNYVV	BSBV
	DAS-ELISA, absorbance, 405 nm	TAS-ELISA, absorbance, 405 nm
Ukr2	0.586 +	0.458 +
Ukr3	0.561 +	0.27 –
Ukr4	0.848 +	0.342 +
Ukr5	1.011 +	0.249 –
Ukr6	0.452 +	0.253 –
Ukr7	0.451 +	0.278 –
Ukr8	0.466 +	0.288 –
Ukr9	0.674 +	0.423 +
Ukr10	0.705 +	0.345 +
Ukr11	0.843 +	0.512 +
Kzt12	0.893 +	0.654 +
K –	0.151	0.176
K +	1.65	2.408

"K–" – negative control; "K+" – positive control; "–" – negative result; "+" – positive result. The ELISA test was positive if absorbance of the investigated sample was equal or greater than 3 times the absorbance of the negative (healthy plant) control (Clark and Adams, 1977).

Identification of BSBV was also confirmed by mRT-PCR. Three specific PCR primer pairs (Meunier et al., 2003), amplificating 291 bp (BVQ), 399 bp (BSBV) and 545 bp (BNYVV) products were used for mRT-PCR (Fig. 15.).

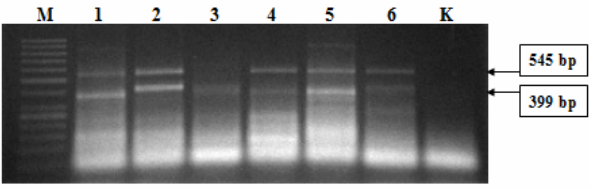

Fig. 15. mRT-PCR products of investigated BNYVV and BSBV mix infection in 2 % agarose gel: M – GeneRuler 50 bp DNA Ladder; 1 – ukr2; 2 – ukr4; 3 – ukr9; 4 – ukr10; 5 – ukr11; 6 – kzt12; K – water control.

In all cases specific BNYVV (545 bp) and BSBV (399 bp) products were obtained (Fig. 15.). mRT-PCR confirmed the ELISA results. BVQ was not found in any investigated sample.

Preparation of polyclonal antiserum to BNYVV and its application for immunodiagnostics. Virus purification. Locally infected indicator plants (*C. quinoa, C. amaranticolor*, *T. expansa*) were used for maintenance of BNYVV. The best results of BNYVV purification were obtained from infected *C. quinoa* leaf tissue using slightly modified method described by Bouzoubaa (1998). The virus was concentrated twice by PEG, NaCl and was purified by high speed ultracentrifugation through 20% sucrose cushion and in 5-45% sucrose density gradient centrifugation. EM was used in BNYVV clarification, concentration and purification stages of purified virus preparations (Fig. 16-18.)

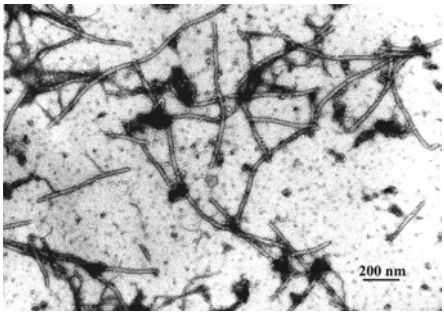

Fig. 16. EM (magnification 20000 ×): BNYVV particles after concentration by PEG.

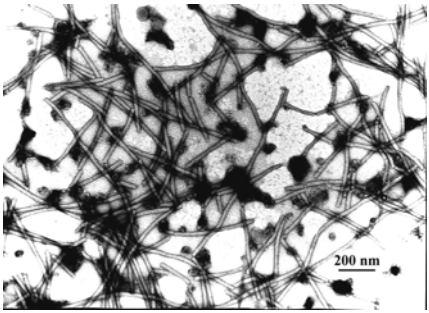

Fig. 17. EM (magnification 25000 ×): BNYVV particles after I ultracentrifugation with 20 % sucrose cushion.

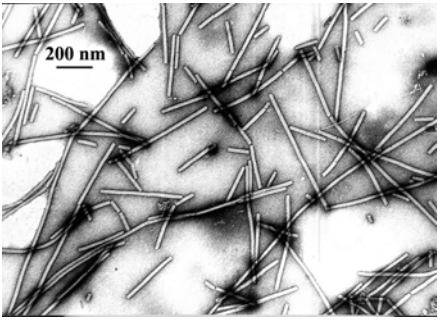

Fig. 18. EM (magnification 20000 ×): particles of purified BNYVV suspension.

Purified virus suspension was analyzed by EM and Western blot (Žižytė and Staniulis, 2007b). EM of purified viral preparation showed straight rod-shaped virus particles typical for rhizomania agent (Fig. 18.) (Richards and Tamada, 1992; Fauquet et al., 2005).

Concentration and yield of obtained virus preparation was evaluated. For estimation of purified virus concentration absorption of viral suspension spectrum (240, 260 and 280 nm) was measured. BNYVV concentration (C) was established according to the formula:

$$C\ (mg/ml) = OD_{260} \times dilution/3.2$$

the number 3.2 is a virus extinction coefficient (Putz and Kuszala, 1978; Bouzoubaa, 1998).

After assessment of viral absorption spectrum and estimation of absorbance at 260 nm the yield of purified BNYVV preparations was 0.237-0.515 mg from 100 g infected leaves material.

Analysis of BNYVV CP. The purified BNYVV suspension was used for analysis of virus CP by Western blot (Žižytė and Staniulis, 2007b). A great advantage of Western

blot is that it identifies the virus by two independent properties of its coat protein – molecular weight and serological specificity (Matthews, 1991).

It is known that molecular mass of BNYVV CP is 21 kDa (Putz, 1977). Purified BNYVV suspension was denatured and CP was separated by 12 % separating and 4 % stacking slab SDS-PAGE gel (Laemmli, 1970). NDS-PAGE of purified BNYVV suspension (Fig. 19.) and Western blot analysis (Fig. 20.) showed a single band, ~21 kDa BNYVV CP. In Western blot BNYVV antiserum reacted only with the CP of purified BNYVV virions and did not reacted with any proteins from healthy *C. quinoa* extracts (Fig. 20. line K).

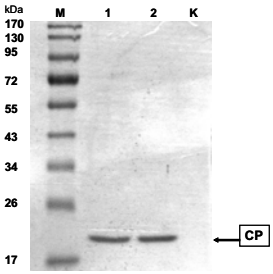

Fig. 19. Analysis of BNYVV CP in SDS-PAGE: M – PageRuler Prestained protein Ladder; 1-2 – purified BNYVV preparation; K – control, extract from healthy *C. quinoa.*

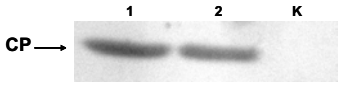

Fig. 20. Western blot analysis of BNYVV CP. 1-2 – purified BNYVV preparation; K – control, extract from healthy *C. quinoa.*

These results confirmed the identification of BNYVV and so purified virus suspension was used for antiserum production, horseradish peroxidase-conjugate preparation and applied for BNYVV diagnostics (Žižytė et al., 2009).

Polyclonal antiserum production. Polyclonal antibody against BNYVV was obtained after rabbit immunization. The antibody sensitivity and the titer were determined using indirect ELISA (Table 8.). The antibodies titer was considered such antibodies dilution in ELISA at which optical density was equal of one. The antibody titer was found to be 1:1600 (Table 8.).

Table 8. Titer determination of polyclonal antiserum against BNYVV in indirect ELISA.

Dilution	Absorption values at 450 nm*
1:200	1.842 (0.048)
1:400	1.669 (0.048)
1:800	1.330 (0.048)
1:1600	0.969 (0.048)
1:3200	0.596 (0.048)
1:6400	0.518 (0.048)
1:12800	0.340 (0.048)
1:25600	0.258 (0.048)
1:51200	0.187 (0.048)
1:102400	0.113 (0.048)

* Average reading of two wells. Value in parentheses is healthy control.

Serological analysis of polyclonal antibodies. In order to develop immunodiagnostics system, polyclonal antibodies were conjugated to horseradish peroxidase (HRP). The obtained pAb-BNYVV HRP conjugate was also checked by titration in direct ELISA (the obtained titer was 1:400) (Table 9.).

Table 9. Titer determination of pAb-BNYVV HPR conjugate in direct ELISA.

Dilution	Absorption values at 450 nm*
1:100	1.329 (0.007)
1:200	1.040 (0.007)
1:400	0.954 (0.007)
1:800	0.593 (0.007)
1:1600	0.330 (0.007)
1:3200	0.165 (0.007)
1:6400	0.105 (0.007)

* Average reading of two wells. Value in parentheses is healthy control.

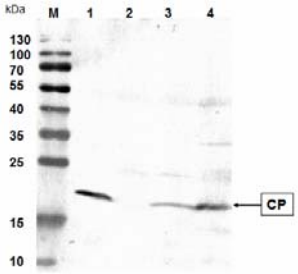

Fig. 21. Western blot analysis of BNYVV CP with pAb-BNYVV HRP. M – PageRuler Prestained Protein Ladder; 1 – purified virus suspension; 2 – an extract from healthy *C. quinoa* (negative control); 3-4 – extracts from infected plant tissues (sugar beet rootlets and *C. quinoa* respectively).

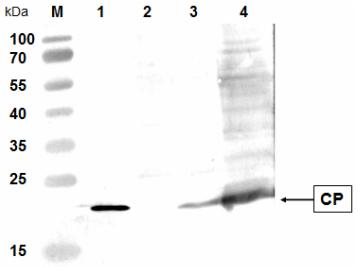

Fig. 22. Western blot analysis of BNYVV CP with pAb-BNYVV. M – PageRuler Prestained Protein Ladder; 1 – purified virus suspension; 2 – an extract from healthy *C. quinoa* (negative control); 3-4 – extracts from infected plant tissues (sugar beet rootlets and *C. quinoa* respectively).

It was found that in direct ELISA the produced pAb-BNYVV HRP conjugate (dilution 1:200) with a different dilution of purified BNYVV suspension (initial concentration of virus suspension 10 µg/ml) could detect BNYVV from 0.3 to 0.15 µg/ml.

The optimal dilutions of pAb-BNYVV (1:1000) and pAb-BNYVV HRP conjugate (1:300) were established to be suitable in immunoenzyme analysis for both ELISA and Western blot.

Diagnostic system for BNYVV was confirmed by Western blot, in which plant samples infected with BNYVV were analyzed. Also specificity of obtained antibodies was verified by Western blot. Purified virus preparation was used as a positive control and a healthy plant tissue – as a negative control (Fig. 21. and 22.).

The pAb-BNYVV HRP conjugate at 1:300 dilution successfully revealed a single band, corresponding to BNYVV CP in purified virus suspension (Fig. 21. 1 line) and in rhizomania infected plant samples (Fig. 21. 3 and 4 lines).

The obtained pAb-BNYVV at 1:1000 dilution with commercial secondary antibodies (anti-rabbit IgG Alkaline Phosphatase conjugate) also gave a single band – BNYVV CP in Western blot analysis (Fig. 22). BNYVV antiserum did not react with

any proteins from crude extract of healthy *C. quinoa* (negative control) (Fig. 21. and Fig. 22. 2 line).

In summary, purified virus suspension was used for polyclonal antiserum production, which could be applied for diagnosis of BNYVV using ELISA and Western blot methods. The enzyme horseradish peroxidase was used for development of immunoenzyme system, suitable to detect BNYVV in infected plants. Such obtained system could detect BNYVV from 0.3 to 0.15 µg/ml and was approved by Western blot.

Conclusions

1. *Beet necrotic yellow vein virus* (BNYVV) was identified in Lithuania, Ukraine and Kazakhstan sugar beet samples with rhizomania symptoms using ELISA and molecular methods.

2. Morphological features of detected virions in rhizomania infected plants and in purified virus preparations are characteristic to BNYVV.

3. Phylogenetic analysis of BNYVV isolates coat protein sequences showed that detected Lithuanian BNYVV isolates belong to different virus types: Pn – to A type, while St and T – to B type. It was found that all Ukrainian BNYVV isolates belong to A type, while the isolate from Kazakhstan – to P type.

4. Lithuanian BNYVV isolates contain distinct amino acids tetrads (in 67-70 position) in P25 protein sequence. Phylogenetic analysis of BNYVV isolates P25 sequences showed the correlation between BNYVV isolates coat protein and P25: Lithuanian St and T isolates belong to B type CP isolates with uniform AYHR tetrad motif, Pn – to A type CP isolates with variable tetrad motif.

5. The obtained purified BNYVV preparation is suitable for polyclonal antibody production.

6. Immunoenzyme system consisted of the obtained polyclonal antibodies against BNYVV and conjugate of polyclonal antibodies and horseradish peroxidase is suitable for diagnosis of BNYVV using ELISA and Western blot methods.

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Runkelių rizomaniją sukeliančio viruso identifikacija ir molekulinė charakteristika

Santrauka

Rizomanija yra viena svarbiausių cukrinių runkelių virusinių ligų. Ją sukelia runkelių nekrotinio gyslų pageltimo virusas (*Beet necrotic yellow vein virus,* BNYVV), priskiriamas *Benyvirus* genčiai. BNYVV yra pernešamas dirvožemio plazmodioforomiceto *Polymyxa betae* Keskin. Pagrindiniai rizomanijos simptomai yra cukrinių runkelių šaknų sutankėjimas, kuokštiškumas, žemaūgiškumas, lapų chlorozė, gyslų pageltimas bei nekrozė. Liga lemia didžiulius ekonominius nuostolius, sumažindama cukrinių runkelių derliaus išeigą, pablogindama cukraus kokybę. Be to, ji yra sunkiai kontroliuojama.

Šio darbo metu BNYVV buvo identifikuotas 3-juose Lietuvos, 10-yje Ukrainos ir 1-oje Kazachstano vietovėse. Atrinkti BNYVV izoliatai buvo mechaniškai pernešti į indikatorinius augalus (*Chenopodium quinoa, C. amaranticolor*, *Tetragonia expansa*), reikalingus viruso sukaupimui, gryninimui, saugojimui bei tolesnei analizei. Viruso izoliatų pernešimas į žolinius indikatorinius augalus taip pat buvo patvirtintas DAS-IFA, EM ir PCR metodais.

EM ir IEM pagalba tiriamuosiuose augaliniuose pavyzdžiuose (tiek cukrinių runkelių šaknelėse, tiek indikatoriniuose augaluose, kuriuose mechaniškai buvo perneštas virusas) buvo aptinkamos įvairaus ilgio, apie 20 nm diametro lazdelės formos BNYVV dalelės. EM taip pat buvo naudojama BNYVV valymo, koncentravimo etapuose ir išgryninto viruso preparatų tyrimui. Išgrynintuose viruso preparatuose aptiktų virionų morfologinės savybės buvo būdingos BNYVV.

Įvairūs PCR metodai buvo taikomi BNYVV identifikacijai. Dažniausiai buvo naudojama vieno žingsnio įprastinė AT-PCR, arba serologiškai imobilizuoto viruso AT-PCR. BNYVV aptikimo padidinimui, buvo atliekama ir lizdinė PCR.

Yra žinoma, kad BNYVV turi tris pagrindinius (A, B ir P) tipus, kurie negali būti atskiriami serologiškai. Tačiau panaudojus restrikcijos fragmento ilgio polimorfizmo analizę, daugybinę AT-PCR bei analizuojant viruso apvalkalo baltymo sekas, buvo nustatyta, kad Lietuvoje aptikti BNYVV izoliatai yra priskiriami skirtingiems A ir B viruso tipams; Ukrainoje – A, o Kazachstane – P tipui. Taip pat pagal P25 baltymo sekos analizę buvo nustatyta, kad Lietuvoje aptikti BNYVV izoliatai P25 sekoje turi skirtingas aminorūgščių tetradas (67–70 padėtyje). B tipo St ir T izoliatai turi AYHR tetradą, A tipo Pn izoliatas – AHHG.

Išgrynintas BNYVV preparatas buvo naudojamas ne tik viruso morfologinių savybių tyrimui EM bet ir antikūnų gamybai. Imunizavus triušį išgrynintu BNYVV preparatu buvo gauti poliklonininiai antikūnai. Iš poliklonininių antikūnų ir šių poliklonininių antikūnų krienų peroksidazės konjugato buvo suformuota imunofermentinė sistema, kuri gali būti sėkmingai taikoma BNYVV diagnostikai.

Curriculum vitae

Name	**Marija Žižytė**
Date and place of birth	8 January, 1980, Vilnius, Lithuania
Address	Nature Research Centre Akademijos 2, LT-08412, Vilnius Lithuania Phone: +370 5 2729838 E-mail: marija.zizyte@botanika.lt

Education

2005 – 2009	PhD studies at the Institute of Botany, Vilnius, Lithuania
2004	Master's Degree in Biochemistry, Vilnius University, Lithuania
2002	Bachelor's Degree in Biochemistry, Vilnius University, Lithuania

Professional Experience

2010 Feb. – present	Specialist, Plant Virus Laboratory, Institute of Botany Nature Research Centre, Vilnius, Lithuania
2009 Nov. – 2010 Jan.	Junior researcher, Institute of Botany, Vilnius, Lithuania
2004 – 2005	Research assistant, Plant Virus Laboratory, Institute of Botany, Vilnius, Lithuania

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