

# Preparation of polyclonal antiserum to beet necrotic yellow vein virus and its application for immunodiagnosics

Marija Žižytė<sup>1\*</sup>,

Indrė Kučinskaitė-Kodžė<sup>2</sup>,

Juozas Staniulis<sup>1</sup>

<sup>1</sup> Laboratory of Plant Viruses,  
Institute of Botany,  
Žaliųjų Ežerų 49,  
LT-08406 Vilnius,  
Lithuania

<sup>2</sup> Laboratory of Immunology  
and Cell Biology,  
Institute of Biotechnology,  
V. A. Graičiūno 8,  
LT-02241 Vilnius,  
Lithuania

Sugar beet rhizomania, caused by the beet necrotic yellow vein virus (BNYVV), was recorded in several areas of Lithuania. From symptomatic sugar beet leaves the virus was mechanically transmitted to the indicator plants for maintenance, purification and storage. The virus was maintained in *Chenopodium quinoa* which served as a propagation host for BNYVV. Transmission of the virus to indicator plants was confirmed by the DAS-ELISA, immunosorbent electron microscopy (IEM) and PCR methods. BNYVV purified by PEG and density gradient centrifugation was used for the production of polyclonal antiserum suitable for immunodiagnosics by ELISA and Western blot analysis. The polyclonal antibody titer obtained in indirect ELISA reactions with a purified virus suspension was found to be 1 : 1600. For the immunoenzyme system formation polyclonal antibodies were conjugated to horseradish peroxidase (HPR). The developed system allowed BNYVV detection in the range of 0.3–0.15 µg/ml.

**Key words:** beet necrotic yellow vein virus, ELISA, horseradish peroxidase, Western blot

## INTRODUCTION

BNYVV (typical member of the genus *Benyvirus*) causes rhizomania – an economically important disease of sugar beet (*Beta vulgaris* L. var. *saccharifera*). The virus is transmitted by mechanical inoculation and by a vector, the soilborne plasmodiophorid *Polymyxa betae* Keskin, which survives in infested soil for many years [1]. BNYVV is a single-stranded RNA virus with tubular rod-shaped particles, 20 nm in diameter, and of five modal lengths ranging from 80 to 390 nm. [2]. All isolates of BNYVV contain RNA1–RNA4 species, and some contain the additional RNA5 (found only in France, the UK, Kazakhstan, Japan and China) [3–7]. Rhizomania, first described in Italy [8] and Japan [9], now has a worldwide distribution in most sugar beet growing areas (Europe, Asia and North America) [10, 11].

The main symptoms of the disease are characterized by a reduced sugar beet taproot size with an extensive proliferation of lateral rootlets, which lead to a serious decrease in root yield and sugar content [1, 12]. The devastating nature of the disease, coupled with the lack of effective and acceptable chemical control methods for the vector, has been a constant problem for the sugar beet industry. The development of rhizomania-resistant sugar beet has been a huge success. However, this breeding success may be short-lived with variants of the virus capable of overcoming resistant cultivars [13]. So, reliable testing procedures are necessary to detect the virus and to follow the spread of the disease.

Surveys to detect BNYVV have been regularly carried out in Lithuania since 1998. In recent years, using commercial kits for DAS-ELISA from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), this virus was detected in three locations of Lithuania [14–16]. In this kit, BNYVV antibodies are conjugated with alkaline

\* Corresponding author. E-mail: marija.zizyte@botanika.lt

phosphatase. The aim of our investigation was to obtain a specific polyclonal antiserum against BNYVV and, using the horseradish peroxidase enzyme, to develop a sensitive, rapid and specific immunoenzyme system for the routine detection of BNYVV and suitable for evaluation of Lithuanian BNYVV isolates at the serological level. HRP is a relatively inexpensive enzyme. It has a high substrate activity and functions at pH values close to neutrality [17]. In our system, we used the non-carcinogenic and non-mutagenic 3,3',5,5'-tetramethylbenzidine (TMB) substrate.

## MATERIALS AND METHODS

### Virus purification

Locally infected indicator plants (*Chenopodium quinoa*, *C. amaranticolor*, *Tetragonia expansa*) were used for the maintenance of BNYVV. The best results of BNYVV purification were obtained from infected *C. quinoa* leaf tissue using a slightly modified method described by Bouzoubaa [18]. The virus was concentrated twice by PEG, NaCl and purified by high speed ultracentrifugation through a 20% sucrose cushion and in 5–45% sucrose density gradient centrifugation. The purified virus suspension was used for EM examination, for polyclonal antiserum production and for coat protein (CP) detection by Western blot analysis.

### Polyclonal antiserum production

The 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 dose (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 an 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 the blood clot was centrifuged at 5000× 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.

### Enzyme-linked immunosorbent assay (ELISA)

**Indirect ELISA.** Indirect ELISA was used to estimate the titer of polyclonal antibodies against BNYVV (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. The plate was incubated overnight at +4 °C. The wells were washed with 200 µl/well with ELISA wash buffer (PBS containing 0.1% Tween-20)

and 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 (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 a sufficient colour developed. The reaction was stopped with 50 µl of 3.6% sulphuric acid solution. The absorbance values were read at 450 nm using a microtitre plate reader (Tecan, Groedig, Austria) [19]. The antibody dilution of ELISA at which optical density was equal to unity was considered as the antibody titer.

**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 overnight, 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 in the same way as in indirect ELISA [19].

### Preparation of plant extracts

Plant material (sugar beet leaves or rootlets) was homogenized in PBST (1 ml of PBS per 0.05 g of plant tissue) and clarified by centrifugation at 5000× g for 15 min. After preparation the extracts were stored at –20 °C.

### HRP-conjugate preparation

Horseradish peroxidase (Merck) was conjugated to pAb-BNYVV using a slightly modified method of Wisdom [20]: 5 mg HRP was dissolved in 1 ml of 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 a Sephadex G-25 column in 1 mM sodium acetate buffer (pH 4.5) and added to pAb-BNYVV solution. The pH of the obtained solution was adjusted to 9.5 by adding a 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 1/1. 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 an overnight dialysis against PBS, BSA (Serva) and glycerol (Roth) were added to give a final concentration of 2% and 50%, respectively. HRP-conjugates were stored at –20 °C.

### Western blot for the detection of coat protein

For BNYVV protein analysis, purified virus preparations and crude extracts of indicator plants or sugar beet rootlets were denatured and CP was separated with a 12% separating a 4% stacking slab sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) in duplicate according to the method of Laemmli [21]. One gel was stained with PageBlue™ Protein Staining Solution (MBI Fermentas). For Western blot analysis, the other gel with BNYVV CP was transferred to a PVDF (polyvinylidene fluoride) membrane [22]. An extract from noninfected *C. quinoa* as well as prestained protein ladder, were included in the transfer. 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. The 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 a PVDF membrane (40 mA) for 40 min.

One transfer was incubated for 30–60 min at room temperature with a blocking buffer (PBS with 1% gelatine), then for 1–2 h with pAb-BNYVVV conjugated to HPR 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 a 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 a blocking buffer (PBS with 1% gelatine), then for 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. The anti-rabbit IgG Alkaline Phosphatase conjugate developed in goat (Sigma; 1 : 30.000 dilution) was used as a secondary antibody with shaking for 1 h 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. The colour was developed by immersing the blot in a 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.

## RESULTS AND DISCUSSION

### Virus purification and polyclonal antiserum production

*C. quinoa* leaf tissue with local lesions was checked for the presence of BNYVV by DAS-ELISA using a DSMZ im-

munological kit, for virus particles morphology by IEM and for molecular identity by the PCR method. A symptomatic leaf tissue was collected and used for BNYVV purification. The yield of purified BNYVV preparations was 0.237–0.515 mg from 100 g of infected leaf material, assuming that the extinction coefficient is 3.2 [18]. EM suspension of purified BNYVV showed rod-shaped virus particles of lengths typical of the rhizomania agent [23]. BNYVV coat protein has a molecular mass of 21 kDa [24]. Western blot analysis of purified virus suspension showed a single band, ~21 kDa BNYVV CP [23]. So, a purified virus suspension was used for antiserum production. Polyclonal antibody against BNYVV was obtained after rabbit immunization. The antibody sensitivity and titer were determined using indirect ELISA. The antibody titer was found to be 1 : 1600 (Table 1).

Table 1. Homologous 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.

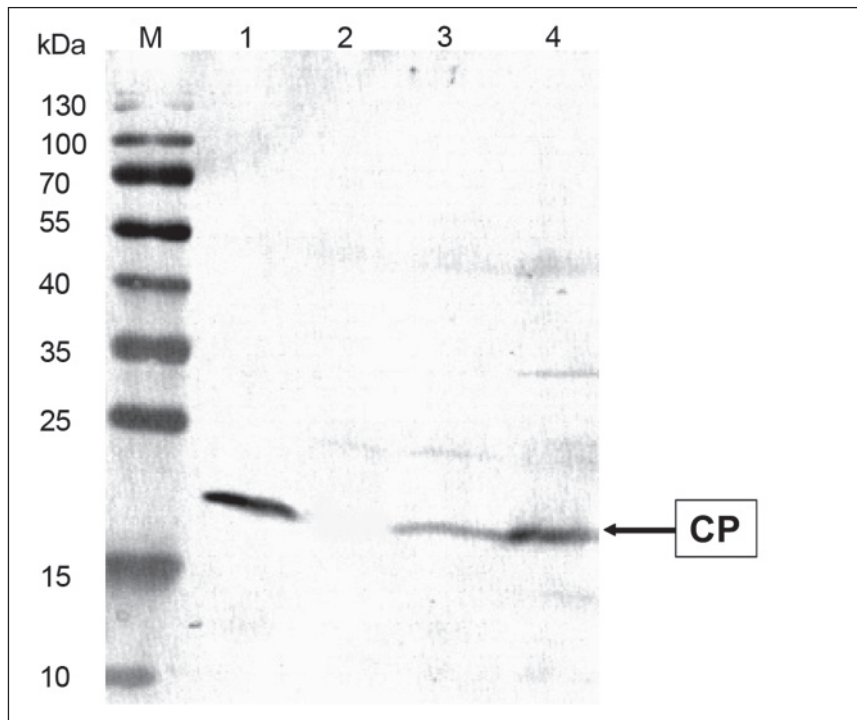
### Serological analysis

In order to develop an immunodiagnostic system, polyclonal antibodies were conjugated to horseradish peroxidase. The obtained pAb-BNYVV HPR conjugate was also checked by titration in direct ELISA (the obtained titer was 1 : 400) (Table 2). The optimal dilutions of pAb-BNYVV (1 : 1000) and pAb-BNYVV HPR conjugate (1 : 300) were established

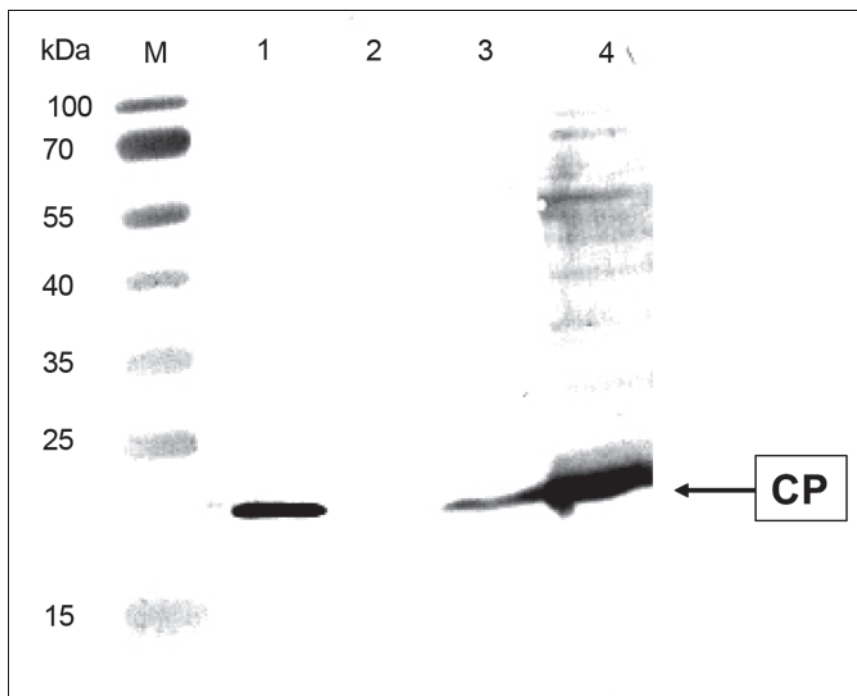
Table 2. Homologous titer determination of pAb-BNYVV HPR conjugate in direct ELISA

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. 1.** Western blot analysis of beet necrotic yellow vein virus coat protein with pAb-BNYVV HPR conjugate. M – molecular masses (kDa) of protein standards (PageRuler™ Prestained Protein Ladder, MBI Fermentas); lane 1 – purified virus suspension; lane 2 – an extract from healthy *C. quinoa* (negative control); lanes 3 and 4 – extracts from infected plant tissues (sugar beet rootlets and *C. quinoa*, respectively)



**Fig. 2.** Western blot analysis of beet necrotic yellow vein virus coat protein with pAb-BNYVV. M – molecular masses (kDa) of protein standards (PageRuler™ Prestained Protein Ladder, MBI Fermentas); lane 1 – purified virus suspension; lane 2 – an extract from healthy *C. quinoa* (negative control); lanes 3 and 4 – extracts from infected plant tissues (sugar beet rootlets and *C. quinoa*, respectively)

to be suitable in immunoenzyme analysis for both ELISA and Western blot.

It was found that in direct ELISA the produced pAb-BNYVV HPR conjugate with a different dilution of purified BNYVV suspension could detect BNYVV from 0.3 to 0.15 µg/ml.

An additional serological test like Western blot confirms the results. The pAb-BNYVV HPR conjugate at a 1 : 300 dilution successfully revealed a single band corresponding to BNYVV CP in the purified virus suspension (Fig. 1). The 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. 2). PAb-BNYVV and pAb-BNYVV HPR conjugate were also used to detect viral antigens in Western blot of infected plant materials. A single immunoreactive band with the molecular weight of 21 KDa was present as detected previously in the purified virus suspension. BNYVV antiserum did not react with any proteins from the crude extract of healthy *C. quinoa* (negative control) (Figs. 1 and 2).

Various immunological assays such as ELISA, IEM, Western blot are used for determining the virus in infected plants. To this end, the Lithuanian BNYVV isolate was purified and used for the production of polyclonal antibodies. The preparation of antiserum and immunoassays for BNYVV detection was reported in several sources [25, 26]. Our study describes attempts to obtain specific polyclonal antibodies against BNYVV and, using horseradish peroxidase, to develop a sensitive immunodiagnostic system employed in routine rhizomania disease screening.

In summary, the data indicate that the developed immunoenzyme system for detecting BNYVV in infected plants has a sufficient sensitivity for virus identification and could be applied for immunodiagnosics because it is suitable for both ELISA and Western blot analysis.

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Marija Žižytė, Indrė Kučinskaitė-Kodžė, Juozas Staniulis

## RUNKELIŲ NEKRO TINIO GYSLŲ PAGELTIMO VIRUSO POLIKLONINIŲ ANTIKŪNŲ PARUOŠIMAS IR JŲ PANAUDOJIMAS IMUNODIAGNOSTIKOJE

### Santrauka

Runkelių nekrotinio gyslų pageltimo virusas (*Beet necrotic yellow vein virus* – BNYVV) yra žalingiausias cukrinių runkelių virusinių ligų sukėlėjas. BNYVV perneša dirvožemio parazitas – *Polymyxa betae* Keskin, kuris išgyvena užkrėstame dirvožemyje daugelį metų ir užkrečia cukrinius runkelius (*Beta vulgaris* var. *saccharifera*) rizomanija. Pagrindiniai rizomanijos simptomai: cukrinių runkelių šaknų sutankėjimas, kuokštiškumas, žemaūgiškumas, lapų chlorozė, gyslų pageltimas bei nekrozė.

Iš trijų rajonų surinktais infekuotais cukrinių runkelių pavyzdžiais mechaniškai buvo inokuliuoti indikatoriniai augalai, siekiant

nustatyti, sukaupti, išgryninti bei išsaugoti virusą. Virusą pernešimui į *Chenopodium quinoa* indikatorinį augalą buvo patvirtintas imunofermen tinės analizės metodu (IFA), imunosorbentine elektronine mikroskopija (ISEM) ir PCR metodais. Grynos ir labai koncentruotos virusų suspensijos gautos diferencinio centrifugavimo metodu. Išgryninta viruso suspensija buvo panaudota polikloninio antiserumo gamybai, kuris galėtų būti taikomas BNYVV diagnostikai IFA ir imunoblotingo metodais. Gautų polikloninių antikūnų titras netiesioginės IFA reakcijose buvo 1 : 1600. Panaudojant krienu peroksidazę buvo sukurta imunofermen tinė sistema, kuri padėjo aptikti BNYVV 0,3–0,15 µg/ml ribose.

**Raktažodžiai:** runkelių nekrotinio gyslų pageltimo virusas, rizomanija, krienu peroksidazė