The Production of Antiserum against *Myrobalan Latent Ringspot Virus* for Detection of the Virus using ELISA

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Abstract

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Myrobalan latent ringspot virus (MLRSV) was purified from the extract of infected *Chenopodium quinoa* Willd., using *n*-octanol, differential centrifugation, and saccharose gradient centrifugation. Electron microscopy showed 28 nm large isometric particles in a sample of purified virus. Antiserum to MLRSV was prepared by immunisation of rabbits with intravenous injections of antigen in combination with intramuscular injections of antigen with Freund's adjuvant. The antibody titer of the obtained antiserum was determined by drop-precipitation method to be 1:1024. Immunoglobulins against MLRSV were isolated from antiserum with ammonium acetate, caprylic acid, and precipitation with ammonium sulphate. The isolated immunoglobulins (IgG) were conjugated with alkaline phosphatase. The optimal dilution of IgG for detection of MLRSV using ELISA was 1×10^{-3} μg/ml which was also the optimal dilution of conjugated IgG. Using this dilution of antibodies, the absorbance of samples from MLRSV-infected plants of *C. quinoa* varied between 0.71 and 1.45, while absorbance of samples from healthy plants (control) was 0.01 to 0.07.

Keywords: nepovirus; MLRSV; purification; immunisation of rabbits; antiserum; IgG isolation; ELISA detection

Myrobalan latent ringspot virus (MLRSV) was first detected and described in France on myrobalan (*Prunus cerasifera* Ehrh.) (DUNEZ *et al.* 1971) and later characterised by DUNEZ and DELBOS (1976). Further research revealed that MLRSV, a nepovirus, is related more to *Cherry leaf roll virus* (CLRV) than to *Tomato black ring virus* (TBRV) (GALLITELLI *et al.* 1981). Specific antibodies against the nepovirus MLRSV have not been prepared so far. Following the introduction of health certification requirements for fruit species intended for planting in the Czech Republic, serological and biological methods to detect the nepoviruses SLRSV (*Strawberry latent ringspot virus)*, CLRV and MLRSV were developed by POLÁK *et al.* (2004). Yet with MLRSV, only biological tests could be used since commercial antibodies for detection of this virus were not available. Therefore, we decided to prepare specific antibodies to be able to detect MLRSV by ELISA.

MATERIAL AND METHODS

Maintenance and propagation of the virus. An isolate of MLRSV obtained from the Mediterranean Agronomical Institute at Bari-Valenzano, Italy,

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was used. The virus was passaged and propagated in plants of *Chenopodium quinoa* Willd. MLRSV was transmitted by mechanical inoculation of plant leaves; 1 g of leaves from infected plants was homogenised in 5 ml 0.066M phosphate buffer, pH 7.1, and the homogenate served as inoculum (Polák *et al.* 2004).

Virus purification. For the immunisation of rabbits, the MLRSV was purified using a modification of the purification method for *Beet yellows virus* (Polák 1972). 100 g leaves from *C. quinoa* systemically infected with MLRSV were homogenised in 0.01M veronal-0.007M phosphate buffer with addition of 0.01M cysteine-hydrochloride and 0.007M EDTA (pH 7.8), 1 g leaves/3 ml buffer. The homogenate was filtered through nylon fabric and centrifuged at 10 000 g for 10 min at 4°C. To the supernatant, 10% saccharose (w/v) and 3% *n*-octanol (v/v) was added. After shaking in a separation cell, the virus was separated from the suspension by centrifugation at 105 000 g for 90 min at 4°C. The chlorophyll containing supernatant was removed and the pellet was resuspended in 2 ml 0.02M phosphate buffer, pH 8.0, and centrifuged at 10 000 g for 5 minutes. The supernatant was then layered in 1ml-volumes on saccharose gradient (10–40% saccharose) and centrifuged at 75 000 g for 120 minutes. Gradients were scanned with ISCO fractionator and the fractions with virus were collected, mixed together, and centrifuged for 150 min at 105 000 g at 4°C. The pellet was resuspended in saline with phosphate buffer, pH 7.0, dialysed against saline, and used for the immunisation of experimental animals.

Immunisation of experimental animals and production of antiserum. The rabbit immunisation was performed by a combination of intravenous injections of antigen and intramuscular injections of antigen with Freund's adjuvant.

Protocol for the immunisation of the rabbit:

Day	Combination of antigen
1	1 ml antigen + 1 ml complete Freund's adjuvant, intramuscularly
7	1 ml antigen + 1 ml incomplete Freund's adjuvant, intramuscularly
14	0.5 ml antigen, intravenously
21	0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant, intramuscularly
49	1 ml antigen + 1 ml incomplete Freund's adjuvant, intramuscularly
63	1 ml antigen, intravenously
77	control blood collection
257	0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant, intramuscularly
276	0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant, intramuscularly
283	0.5 ml antigen, intravenously
297	blood collection

Two rabbits were immunised. Antiserum was gained by separation of the serum from the whole blood. The titer of the antiserum was determined by drop-precipitation method.

RESULTS AND DISCUSSION

The purification of MLRSV from the leaves of systemically infected plants of *C. quinoa*, using differential centrifugation and centrifugation on saccharose gradient, was performed three times. The collected samples were stored as partially purified virus at 4°C and used for the immunisation of rabbits, electron microscopy, and mechanical transmission into *C. quinoa*. The extinction ratio $E_{260/280}$ was 1.7, proving the presence of sufficiently pure nucleoprotein. During the purification process the virus has not lost its biological activity. After 3 months storage at 4°C, the virus was still infective in *C. quinoa*. The preparation was appropriate for contrasting MLRSV particles with sodium phospho-wolframate. On the micrograph (Figure 1), isometric particles of MLRSV of the size 28 nm can be seen. The size and shape of

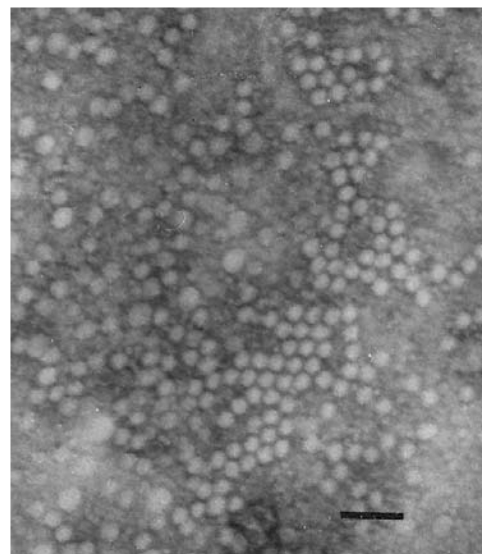

Figure 1. Electronogram of partly purified *Myrobalan latent ringspot virus*, isometric particles 28 nm (direct magnification 23 000×; bar = 100 nm)

these particles correspond to data found in the literature (Brunt *et al*. 1996).

After the immunisation process was completed, antiserum was obtained. Its titer was determined by drop-precipitation method to be 1:1024. The antiserum reacted specifically with an extract from MLRSV-infected *C. quinoa* leaves and with a sample of purified MLRSV. It did not react with an extract from healthy *C. quinoa*.

Specific immunoglobulins (IgG) were isolated from 5 ml of MLRSV antiserum. Sodium acetate and octanoic acid were used for precipitation of impurities. Precipitated components were removed by centrifugation. The supernatant was dialysed against 0.02M potassium phosphate buffer pH 7.2. The antibodies were mixed with saturated ammonium sulphate. The precipitated immunoglobulins were separated by low speed centrifugation. The sediment was diluted in 5 ml of distilled water. Residues of ammonium sulphate were removed by 24 h dialysis against 0.02M potassium-phosphate buffer pH 7.2. The concentration of the antibodies after spectrophotometer measuring was set to 1 mg/ml (A_{280} = 1.4). The prepared IgG of MLRSV specific IgG were stored at minus –20°C.

Immunoglobulins were conjugated with alkaline phosphatase (Fluka, Germany) with enzymatic activity of 3141 U/mg protein. Glutaraldehyde was used at 25% in the procedure described by Polák *et al*. (1993). Finally, bovine serum albumin and sodium azide were added for conservation and the conjugated antibodies were stored at 4°C.

The sap of *C. quinoa* plants infected with MLRSV and partially purified MLRSV were used as antigens to test the prepared antibodies. The sap from healthy plants of *C. quinoa* was used as control. The optimal working concentration of IgG was 1 µg/ml. The optimal dilution of alkaline phosphatase-conjugated IgG was 1:1000. Using the optimal antibody dilutions, the extinction value of samples from MLRSV-infected *C. quinoa* ranged between 0.71 and 1.45, while that of samples from healthy *C. quinoa* (control) ranged between 0.01 and 0.07.

The results proved that the produced MLRSV antiserum and the isolated antibodies are suitable for detection of the nepovirus MLRSV in infected plants using ELISA. MLRSV was detected in woody hosts with prepared antibodies.

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