Biological and Serological Procedures to Detect Three Nepoviruses in Fruit Trees

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Abstract

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Cherry leaf roll virus (CLRV), *Myrobalan latent ringspot virus* (MLRSV) and *Strawberry latent ringspot virus* (SLRSV) were transferred by budding to woody trees, hybrid Ishtara, peach cv. GF 305 and cv. Lesiberian. Three buffers with antioxidants and stabilisers: 0.01M phosphate with 1% caffeine; 0.007M phosphate-0.01M veronal with 0.01M cysteine hydrochloride and 0.007 EDTA; 0.015M phosphate with 1% nicotine and 0.066M phosphate buffer without additives were compared for their efficiency in mechanical transmission from woody sources to herbaceous hosts (*Chenopodium quinoa* and *C. amaranticolor*). 0.007M phosphate-0.01M veronal buffer with 0.01M cysteine hydrochloride, and 0.007 EDTA and 0.015M phosphate buffer with 1% nicotine were found to be the best buffers for the three nepoviruses. Both biological transmission to herbaceous assay hosts and detection by ELISA in the investigated tree are necessary to reliably detect the three nepoviruses. Biological detection is reliable from April to June, and in September and October. ELISA detection is also more difficult in July and August. The suitability of *C. quinoa* and *C. amaranticolor* to maintain CLRV, MLRSV and SLRSV was compared. *C. amaranticolor* plants were found to be more suitable for CLRV and SLRSV, infected plants grow over 6 months after mechanical inoculation by the nepoviruses. *C. quinoa* plants proved to be most suitable for maintenance of MLRSV, while *C. amaranticolor* is a symptomless host of MLRSV. Reinoculation with the nepoviruses is recommended in intervals of 4 to 6 months.

Keywords: *Cherry leaf roll virus*; *Myrobalan latent ringspot virus*; *Strawberry latent ringspot virus*; mechanical transmission; herbaceous hosts; bud transmission; fruit trees; DAS-ELISA; electron microscopy

Nepoviruses infect different species of stone fruit trees and cause serious diseases and death of infected trees. *Cherry leaf roll virus* (CLRV) was proved in trees of *Prunus avium*, *P. cerasifera*, *P. persica* and *Juglans regia*. *Myrobalan latent ringspot virus* (MLRSV) has been found only in France (Dunez & Delbos 1976); it has a limited natural host range, infecting *Prunus avium*, *P. cerasifera* and *P. persica*. The widely spread *Strawberry latent ringspot virus* (SLRSV), with a broad natural host range, has been reported first in *Fragaria vesca* and *Rubus idaeus* (Lister 1964), and was later proved in *Prunus domestica*, *P. persica*, *Vitis vinifera*, *Ribes* spp. and many other hosts. Suitable assay hosts of CLRV, MLRSV and SLRSV are *Chenopodium quinoa* and *C. amaranticolor* (Brunt *et al*. 1996). A common method for detection of CLRV (Mircetich *et al*. 1980) and SLRSV (Thomas 1980) is ELISA.

For Czechoslovakia, the presence of CLRV was proved in cherry trees (Albrechtová & Chod 1981), in walnut (Novák & Lanzová 1981) and in various cultivars of raspberry (Janečková & Pluhař 1987).

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SLRSV was proved in cherry trees by Novák and Lanzová (1975). Křístek and Polák (1990) used ELISA for detection of SLRSV in grapevine (*Vitis vinifera*) cultivars in Bohemia (district Mělník), and in some herbaceous plants. The detection of nepoviruses in fruit trees is not easy because of the often low concentration of virus in plant tissues. Many publications deal with the detection of nepoviruses in plants, but there has been no research on the reliability of detection in fruit trees during the vegetation period. Yet suitable and reliable standard methods for the detection of CLRV, MLRSV and SLRSV in stone fruit trees are necessary for a proper and meaningful certification of planting material of fruit trees in the Czech Republic.

MATERIAL AND METHODS

Nepoviruses, bud transmission to woody hosts. Isolates of CLRV, MLRSV and SLRSV in grafts of peach, cv. GF-305, were obtained from the Mediterranean Agronomical Institute, Bari-Valenzano, Italy. Virus free trees of Ishtara (hybrid of *Prunus cerasifera* × *P. japonica* × *P. persica*) and peach, cv. GF-305 or cv. Lesiberian, were inoculated by chip-budding with CLRV, MLRSV and SLRSV using infected buds. The presence of a specific nepovirus in inoculated trees of Ishtara and peach cv. GF-305 or cv. Lesiberian was verified by ELISA (CLRV, SLRSV), and by mechanical transmission to the herbaceous host *Chenopodium quinoa* (CLRV, SLRSV, MLRSV). Commercial antibodies from Löwe (BRD) were used in DAS-ELISA.

Mechanical transmission of nepoviruses from woody to herbaceous host. Tests were conducted to find a reliable method of mechanical transmission of CLRV, MLRSV and SLRSV from woody hosts to herbaceous assay hosts. Four different buffers were used for mechanical transmission of the three nepoviruses from woody to the herbaceous hosts *C. quinoa* and *C. amaranticolor*:

1. 0.007M phosphate-0.01M veronal buffer with 0.01M cysteine hydrochloride and 0.007M EDTA, pH 7.8;
2. 0.015M phosphate buffer with 1% nicotine, pH 8.5;
3. 0.02M phosphate buffer with 0.01 M DIECA, 0.01M diphenyl dithiourea and 1% caffeine, pH 8.6;
4. 0.066M phosphate buffer, pH 7.1.

The influence of different buffers on infectivity of the homogenate obtained from leaves of infected woody hosts was determined. Local lesions on the herbaceous hosts were counted and the intensity of systemic symptoms evaluated. The presence of the nepoviruses in these hosts was checked by ELISA and electronmicroscopic examination.

Maintenance of viruses in herbaceous assay hosts. CLRV and SLRSV were maintained in plants of *C. amaranticolor*, MLRSV was maintained in *C. quinoa*. One gram of infected leaves was homogenised with 5 ml of 0.066M phosphate buffer, pH 7.1, and used for mechanical inoculation of young plants of *C. amaranticolor* or *C. quinoa* that had six to eight fully developed leaves. Plants were decapitated 24 h before inoculation.

Reliability of biological and serological (ELISA) detection of nepoviruses in woody hosts. The reliability of detection of CLRV, MLRSV and SLRSV in woody hosts, i.e. trees of Ishtara and peach cv. GF-305 or cv. Lesiberian, was tested every month during the whole vegetation period. One gram of leaves from an infected tree was homogenised in 5 ml of 0.015M phosphate buffer with 1% nicotine, pH 8.5, and used for mechanical inoculation of three plants of *C. amaranticolor*. Assay plants were decapitated and placed in darkness for 24 h before mechanical inoculation. The presence or absence of virus symptoms in an inoculated *C. amaranticolor* plants was evaluated after the incubation period.

The presence of CLRV and SLRSV in leaves of woody hosts was parallel tested by ELISA. Commercial antiserum from Löwe (BRD) was used. 0.2 g of leaves was homogenised in 5 ml of extraction buffer, PBS + 1% PVP + 0,1% bovine serum albumin, pH 7.4. Commercial antibodies of MLRSV are not available.

Electron microscopy. A drop of homogenate from a leaf of a herbaceous or woody plant systemically infected with nepovirus was placed on a Formwar-coated carbon grid, stained with 4% phosphotungs acid pH 6.9 and examined with a Philips EM 208 HS electron microscope.

RESULTS AND DISCUSSION

Only few trees of Ishtara and peach cv. GF-305 or cv. Lesiberian became infected with CLRV, MLRSV and SLRSV after inoculation by budding. The incubation period was 3–6 months. Ten trees of Ishtara and two trees of peach cv. Lesiberian were inoculated with CLRV, but only one tree of cv. Lesiberian became infected and none of Ishtara. Leaves of the infected tree of cv. Lesiberian showed only very mild

spots. CLRV was proved in cv. Lesiberian by ELISA and by mechanical transmission to *C. quinoa* and *C. amaranticolor*. Trees of Ishtara were reinoculated with CLRV after 2 years. The presence of CLRV was proved after 3 months by ELISA.

Eight trees of Ishtara and two of cv. GF-305 were inoculated with MLRSV. Two trees of Ishtara were infected, but none of cv. GF-305. Some leaves of infected trees showed small spots and rings (Figure 1). Every mechanical transmission of MLRSV from a tree of Ishtara to herbaceous hosts was successful.

Nine trees of Ishtara and one of cv. Lesiberian were inoculated with SLRSV. Six trees of Ishtara and one of cv. Lesiberian became infected; two trees of Ishtara died several months after infection with SLRSV, and the tree of peach cv. Lesiberian died 2 years after infection. The presence of SLRSV in infected trees was proved by DAS-ELISA and by mechanical inoculation to *C. quinoa* and *C. amaranticolor*. The presence of the virus in the herbaceous hosts was confirmed by DAS-ELISA.

Two buffers with antioxidant and stabilisers (veronal-phosphate buffer with cysteine hydrochloride and EDTA, and phosphate buffer with 1% nicotine) showed a higher efficiency of mechanical transmission from woody to herbaceous host than phosphate buffer without additives (Table 1). In trials with SLRSV the phosphate buffer with nicotine gave the best results, and high absorbance values were measured in samples from infected *C. quinoa* plants. Three buffers with antioxidants and stabilisers were compared in other trials with mechanical transmission of CLRV and MLRSV from woody to herbaceous hosts (Table 2). Not all inoculated plants of *C. quinoa* became systemically infected. Veronal-phosphate buffer with cysteine hydrochloride, and phosphate buffer with 1% nicotine resulted in higher efficiency of mechanical transmission of CLRV and MLRSV to *C. quinoa*. Lower efficiency of mechanical transmission of both nepoviruses was obtained when phosphate buffer with caffeine, diphenyldithiourea and DIECA was used. Thus, phosphate buffer with 1% nicotine, or veronal-phosphate buffer are recommended for biological detection of CLRV, MLRSV and SLRSV in woody hosts.

The suitability of the herbaceous assay hosts *C. quinoa* and *C. amaranticolor* to maintain CLRSV, MLRSV and SLRSV was compared. CLRV and SLRSV were mechanically transmitted to plants of *C. quinoa* and *C. amaranticolor* with 100% efficiency following the procedure described above and by using 0.066M phosphate buffer (Sörensen) pH 7.1. Phosphate buffer with pH 7.0 and 7.2 had been successfully used in mechanical transmission of nepoviruses in herbaceous hosts (e.g. Jones & Mayo 1972; Gentit *et al.* 2001). *C. quinoa* and *C. amaranticolor* reacted with local lesions and systemic infection to CLRV and SLRSV, and are the best assay hosts for these viruses. The systemic reactions of plants of these two hosts to infection by CLRV is vein clearing, vein banding, apical necrosis, discoloration, malformations and stunting of leaves. These symptoms appeared within 10–14 days after inoculation. *C. quinoa* plants with systemic symp-

Table 1. Comparison of the efficiency of phosphate buffer with two buffers containing antioxidants for the mechanical transmission of MLRSV and SLRSV from woody to herbaceous host

Virus	Source of virus (woody host)	Buffer for transmission	Herbaceous host	Number of inoculated plants (à 6 leaves)	Average number of LL/leaf
MLRSV	Ishtara	4. phosphate	*Chenopodium quinoa*	18	0.27
		1. veronal-phosphate		18	0.33
		2. phosphate + nicotine		18	0.31
SLRSV (trial 1)	Ishtara	4. phosphate	*Chenopodium quinoa*	18	0.49
		1. veronal-phosphate		18	0.77
		2. phosphate + nicotine		18	1.05
SLRSV (trial 2)	Ishtara	4. phosphate	*Chenopodium quinoa*	18	0.52
		1. veronal-phosphate		18	0.77
		2. phosphate + nicotine		18	1.57

Figure 1. Leaves of Ishtara with systemic symptoms of *Myrobalan latent ringspot virus*

Figure 2. *Chenopodium quinoa* plants with local lesions and systemic symptoms of *Myrobalan latent ringspot virus*

toms of SLRSV are shown in Figure 3. Symptoms on *C. amaranticolor* infected with CLRV were the same, but symptoms caused by SLRSV were different, more discoloured. A plant of *C. amaranticolor* systemically infected with SLRSV is compared with healthy plants in Figure 4.

C. quinoa plants proved to be most suitable for maintenance of MLRSV. Symptoms of vein clearing, vein banding, apical necrosis, malformations and stunting of leaves are very similar to symptoms of CLRV and SLRSV, but the virus can be maintained for a longer time, at least 4 months. *C. quinoa* plants with local lesions and systemic symptoms of MLRSV are presented in Figure 2. *C. amaranticolor* is a symptomless host of MLRSV. No systemic symptoms appeared in leaves of *C. amaranticolor* after mechanical inoculation by MLRSV, but it is possible to transmit the virus to *C. quinoa* plants.

Figure 3. *Chenopodium quinoa* plants with systemic symptoms of *Strawberry latent ringspot virus*

Table 2. Evaluation of three buffers used in the mechanical transmission of CLRV and MLRSV from woody to herbaceous host

Virus	Source of virus (woody plant)	Buffer for transmission	Herbaceous host	Number of inoculated plants	Number of systemically infected plants
CLRV (trial 1)	Ishtara	1. veronal-phosphate	*Chenopodium quinoa*	6	3
		2. phosphate + nicotine		6	4
		3. phosphate + caffeine		6	1
CLRV (trial 2)	Ishtara	1. veronal-phosphate	*Chenopodium quinoa*	6	5
		2. phosphate + nicotine		6	4
		3. phosphate + caffeine		6	2
MLRSV	Ishtara	1. veronal-phosphate	*Chenopodium quinoa*	6	6
		2. phosphate + nicotine		6	6
		3. phosphate + caffeine		6	3

Figure 4. Plant of *Chenopodium amaranticolor* with systemic symptoms of *Strawberry latent ringspot virus* (on the left); healthy control plants (on the right)

C. amaranticolor plants were found to be more suitable for maintenance of CLRV and SLRSV. *C. quinoa* plants died usually within 1–3 months after mechanical inoculation, while systemically infected *C. amaranticolor* plants grew well for more than half a year after mechanical inoculation (Figure 5). Reinoculation with CLRV and SLRSV is recommended after an interval of 4 to 6 months.

Figure 5. *Chenopodium amaranticolor* plant systemically infected with CLRV seven months after mechanical inoculation (on the right); healthy control plant (on the left)

In trials on biological (CLRV, MLRSV, SLRSV) and serological (CLRV, SLRSV) detection in woody hosts it was found that it is possible to detect nepovirus in some trees by mechanical inoculation to *C. quinoa* and *C. amaranticolor* from April to June and from September to October. Detection by ELISA is more difficult in July and August. CLRV was detected in peach, cv. Lesiberian, by mechanical inoculation to *C. quinoa* and *C. amaranticolor* from April to June, and in September and October. CLRV was detected in cv. Lesiberian by ELISA for the whole vegetation period. MLRSV was detected in one tree of Ishtara by mechanical inoculation to *C. quinoa* throughout the whole vegetation period. In another tree of Ishtara, MLRSV was detected by mechanical inoculation to *C. quinoa* only in some months during the vegetation period. MLRSV could not be detected by ELISA because commercial antibodies are not available. Particles of MLRSV were observed in leaves of Ishtara by electron microscopy (Figure 6.) SLRSV was detected in one peach tree, cv. Lesiberian, by inoculation to *C. quinoa* and by ELISA during the whole vegetation period. SLRSV was detected in one tree of Ishtara by mechanical inoculation to *C. quinoa* and *C. amaranticolor* and by ELISA in several months during the summer. In further two trees of Ishtara, biological tests sometimes revealed the presence of SLRSV by indicator plants, but its presence in the woody host was not confirmed by ELISA. Two plants of Ishtara infected with SLRSV died in the year of inoculation. A peach tree of cv. Lesiberian infected with SLRSV died 2 years after inoculation (Figure 7). SLRSV was detected throughout

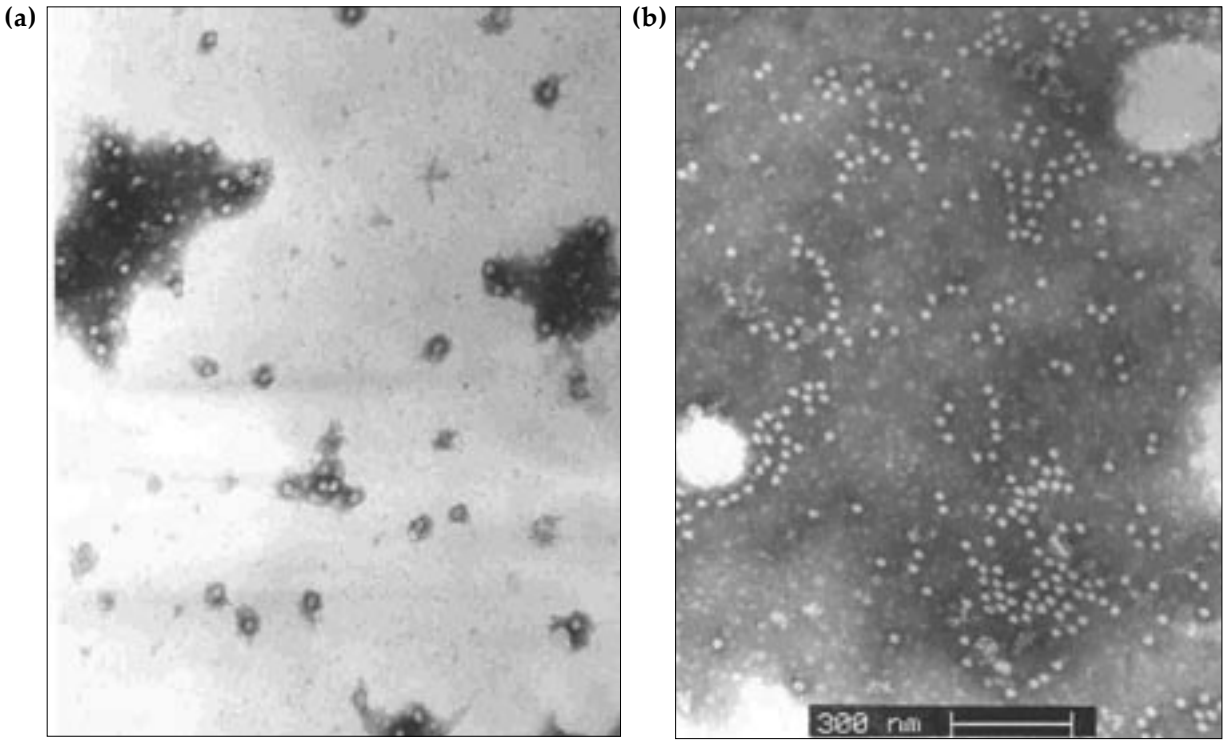

Figure 6. Electronogram of MLRSV particles (a) Individual particles of MLRSV in homogenate from leaves of infected tree Ishtara; (b) Particles of MLRSV in partial purification from infected *Chenopodium quinoa*

the whole vegetation period by biological and serological tests in this tree.

Tests on the reliability of biological (for CLRV, SLRSV and MLRSV) and serological, i.e. ELISA (for CLRV SLRSV) detection of nepoviruses in woody hosts proved that both biological transmission to herbaceous assay hosts and ELISA detection in the woody hosts are necessary for reliable detection of the presence of nepovirus in an investigated tree.

Figure 7. Dead tree of *Prunus persica*, cv. Lesiberian, after two and half years infection with SLRSV

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Souhrn

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Virus svinutky třešně (CLRV), virus latentní kroužkovitosti myrobalánu (MLRSV) a virus latentní kroužkovitosti jahodníku (SLRSV) byly přeneseny očkováním na dřeviny: Ishtara (hybrid *Prunus cerasifera* × *P. japonica* × *P. persica*), broskvoň, cv. GF 305 a cv. Lesiberian. Tři pufry s antioxidanty a stabilizátory (0,01M fosfátový pufr s 1 % kofeinu; 0,007M fosfátový pufr s 0,01M veronalem, 0,01M cystein hydrochloridem a 0,007M EDTA; 0,015M fosfátový pufr s 1% nikotinem) byly porovnávány s 0,066M fosfátovým pufrem bez aditiv při mechanických přenosech z dřevinných na bylinné hostitele (*Chenopodium quinoa* a *Chenopodium amaranticolor*). 0,007M fosfátový pufr s 0,01M veronalem, 0,01M cysteinhydrochloridem a 0,007M EDTA a 0,015M fosfátový pufr s 1% nikotinem byly vyhodnoceny jako nejlepší pro všechny testované nepoviry. Obě metody, biologický přenos na bylinné indikátory i stanovení pomocí ELISA, jsou nutné pro spolehlivé určení těchto tří nepovirů. Biologická detekce nepovirů je spolehlivá od dubna do června a od září do října. Detekce pomocí ELISA je také těžší v červenci a srpnu. Byla ověřována i vhodnost *C. quinoa* a *C. amaranticolor* pro udržování CLRV, MLRSV a SLRSV. Druh *C. amaranticolor* byl určen jako vhodnější pro CLRV a SLRSV, protože infikované rostliny tohoto druhu rostou více než 6 měsíců po mechanické inokulaci nepoviry. Jako nejvhodnější pro udržování MLRSV byly prokázány rostliny *C. quinoa*, *C. amaranticolor* je bezpříznakovým hostitelem viru. Reinokulace virů se doporučuje v intervalu 4–6 měsíců.

Klíčová slova: virus svinutky třešně; virus latentní kroužkovitosti myrobalánu; virus latentní kroužkovitosti jahodníku, mechanický přenos; bylinné indikátory; přenos očkováním; ovocné stromy; DAS-ELISA; elektronová mikroskopie

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