BIOLOGIJA. 2005. Nr. 2. P. 29–34

Viral diseases of flower plants 16. Identification of viruses affecting orchids (*Cymbidium* Sw.)

M. Navalinskienë[1],

J. Raugalas[2],

M. Samuitienë[1]

[1] *Plant Virus Laboratory, Institute of Botany, Þaliøjø eþerø 49, LT-2021 Vilnius, Lithuania*
[2] *Department of Plant Physiology and Microbiology, Vilnius University, M. K. Èiurlionio 21/27, LT-2009 Vilnius, Lithuania*

Two viruses, *Cymbidium mosaic potexvirus* (CymMV) and *Odontoglossum ringspot tobamovirus* (ORSV), were isolated and identified from *Cymbidium* Sw. plants in Lithuania. Viruses were identified by the methods of test-plants and electron microscopy. The optimum techniques for purification of the viruses were selected and the purified preparations (antigens) were obtained. The yield of purified preparations was 7.5 mg from 100 g of infected plant tissue for CymMV and 12.4 mg for ORSV. The titres of the produced specific diagnostic antisera in DAS-ELISA test were 1: 2560 to CymMV and 1: 40960 to ORSV. The IgGs from antisera were precipitated and purified. Specific enzyme-labeled ORSV antibodies (conjugates) with horseradish peroxidase and inorganic pyrophosphatase of *Escherichia coli* were prepared and estimated. The possibly optimal conditions for the DAS-ELISA test were developed. Our experiments showed the possibility and advantages of using inorganic pyrophosphatase of *Escherichia coli* in the DAS-ELISA system.

Key words: *Cymbidium* Sw., *Cymbidium mosaic potexvirus, Odontoglossum ringspot tobamovirus,* identification, DAS-ELISA

INTRODUCTION

Cymbidium Sw. (*C. hybridum*) belongs to the family of *Orchidaceae* Juss. The *Orchidaceae* is the largest and the most diverse plant family, consisting of more than 25000 species in approximately 900 genera. An equally large number of orchid hybrids exists, many of which are multigenic. Orchids grow naturally in a wide range of habitants ranging from temperate through tropical climates in many parts of the world. Grown commercially in many different countries, orchids have perhaps the highest unit value of any ornamental plants. The procedure of mericloning developed in the 1960s greatly reduced the cost of orchid plants, and as a result, culture expanded rapidly. The market for orchid plants and flowers has expanded as new hybrids have been introduced. Cut flower production has been concentrated in Asia and Hawaii, in the US [1].

Orchids of the genus *Cymbidium* were first described by Swedish botanist O. Swartz in 1799. *Cymbidium hybridum* is a complex intergeneric hybrid. The first hybrid was produced in England in 1889 [2]. A tetraploid hybrid on whose basis modern *Cymbidium* cultivars have been produced was made in 1947 [3]. Breeding for *Cymbidium* Sw. cultivars is concentrated in the USA.

In Lithuania, *Cymbidium hybridum* has been commercially grown since the 1980s. Plant material was obtained from the USA, the Ukraine, Latvia, and Russia.

Orchids, more than most crops, are affected by virus diseases reducing their commercial value considerably. More than 27 viruses are described to affect this crop [1]. Orchid viruses are widespread in cultivated orchids, *Cymbidium mosaic potexvirus* (CymMV) and *Odontoglossum ringspot tobamovirus* (ORSV) being the most prevalent and economically important. CymMV causes chlorotic or necrotic sunken patches on leaves and necrosis on flowers; ORSV induces necrotic rings on leaves and colour breaking and distortion on flowers [4, 5]. Both viruses reduce plant vigour, growth rate, an flower quality [6]. They are not transmitted by natural vectors but spread by contaminated tools and pots during division of plants and harvest of flowers [5]. Both viruses are stable and mechanically transmissible [7]. CymMV affects other orchid genera (*Cattleya* Ldl., *Dendrobium* Sw., *Epidendrum* L., *Laelia* Ldl., *Phaleonopsis* Bl., *Vanda* sp.) and ORSV damages *Epidendrum, Odontoglossum* H. B. et Kth., *Oncidium* Sw. [1].

The aim of this work was to identify viruses affecting *C. hybridum*, to prepare ORSV and CyMV

purified preparations (antigens), to produce specific diagnostic antisera and to develop the possibly optimal conditions for DAS-ELISA test.

MATERIALS AND METHODS

Material for investigation was collected in different orchid nurseries in Lithuania: Botanical Gardens of Vilnius University, Floriculture Department of Horticulture (farm in Kaišiadorys), Floriculture farm 'Panerys' (Vilnius), and the Vilnius greenhouse enterprise. Samples were collected from *Cymbidium plants* expressing viral symtoms on leaves and flowers. The experimental work was carried out at the greenhouse and Laboratory of Plant Viruses of Institute of Botany. Viruses have been identified by test-plant reaction [8, 9] and by virus particle morphology. Virus particles were visualized in negatively stained dip preparations [10] using a JEM-100S electron microscope.

For mechanical sap inoculation the following test-plants were used: *Beta vulgaris* L. var. circla, *Chenopodium amaranticolor* Coste et Reyn, *C. quinoa* Willd., *Datura stramonium* L., *D. metel* L., *Gomphrena globosa* L., *Nicandra physalodes* L., *Nicotiana glutinosa* L., *N. tabacum* L. 'Samsun', 'Xanthi', *Petunia hybrida* Vilm., *Phaseolus vulgaris* L. 'Prince', *Tetragonia expansa* Murr. The inoculum for mechanical inoculation was prepared by homogenizing infected leaves with 0.1 M phosphate buffer (pH 7.0) containing virus stabilizing agents – 1% sodium bisulphite and 0.01 M sodium diethyldithiocarbamate (DIECA).

For purification, the virus isolates after two successive single lesion transfers on *Datura stramonium* (for CymMV) and on *Gomphrena gobosa* (for ORSV) were maintained in *Chenopodium quinoa* expressing necrotic local lesions on inoculated leaves.

CyMV was purified from *C. quinoa* leaves according to a modified Frowd and Tremaine method [11]. Fresh leaves were homogenized with 0.2 M boracic buffer at pH 8.0 (3 ml/g tissue) containing 0.1% 2-mercaptoethanol and 0.1% DIECA. The homogenate was clarified with a chloroform / butanol mixture (1:1) to a final concentration of 8.5%. The virus was precipitated from the aqueous phase with polyethylene glycol MW 6000 (PEG_{6000}) (4 g/100 ml) with NaCl (0.2 g/100 ml). The pellets were resuspended in 0.2 M boracic buffer (pH 8.0) and centrifuged for 10 min at 8000 g. After two cycles of differential centrifugation (the supernatant was centrifuged over a 30% sucrose cushion for 1.5 h at 80000 g (rotor SW3 × 35; centrifuge VAC-601) and the pellets were resuspended in 0.1 M boracic buffer at pH 8.0 and centrifuged for 10 min at 8000 g) the pellets were resuspended in 0.01 M boracic buffer pH 8.0 with 0.01% sodium azide.

ORSV was purified from *C. quinoa* leaves, which showed necrotic local lesions 15 days after inoculation. Fresh leaves were homogenized with 0.2 M boracic buffer at pH 8.0–8.2 (3 ml/g tissue) containing 0.005 M EDTA and 0.01 M DIECA. The homogenate was clarified with a chloroform / butanol mixture (1:1) to a final concentration of 8.5%. The virus was precipitated from the aqueous phase with polyethylene glycol MW 6000 (PEG_{6000}) (6 g/100 ml) with NaCl (0.2 g/100 ml). Further purification procedures were acomplished as described for CyMV.

The yields of purified viruses were determined spectrophotometrically, taking into account the specific absorbance $A_{0.1\ \%/260\ nm}$ 3.0 for ORSV [12, 13] and 2.97 for CymMV [14, 15].

Antisera to ORSV and CymMV were produced by injecting rabbits three times intracutaneously with 0.5 mg purified virus mixed with 1 ml Freund's complete adjuvant at one-week intervals. Blood was collected 2 weeks after the last injection. The titre was determined in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA test).

The immunoglobulin (IgG) fraction was purified by precipitation with ammonium sulfate [16] at 40% saturation and subsequent passage through a diethylaminoethyl cellulose column DE 52 (Sigma).

The conjugates were prepared by coupling purified IgGs with horseradish peroxidase and inorganic pyrophosphatase of *Escherichia coli* according to the Baykov, Kasho and Avaeva method [17].

DAS-ELISA systems were prepared from our conjugates. Colour development was achieved using the substrate – sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) with malachite green and Na molybdate. Colour development was monitored at 620 nm using an AIF-C-01S plate reader.

RESULTS AND DISCUSSION

A survey of *Cymbidium* Sw. plants in orchid nurseries revealed the presence of main symptoms of virus diseases: mosaic, rhombus-shaped and necrotic streaking, mottling on leaves and necrotic flecking on flowers.

All collected samples were examined by electron microscopy. It was revealed that flower necrotic flecking symptoms were induced by a virus with flexuous filamentous particles approximately 480 nm long. The straight rod-shaped particles approximately 300 nm in length were revealed in plants expressing rhombus-shaped streaking and mottling. Necrotic streaking symptoms were induced by both types of virus particles but more commonly by filamentous virions. According to data of test-plant reactions and virus particle morphology, two viruses affecting *Cymbidium* plants were identified.

Cymbidium mosaic potexvirus. Symptoms on young leaves were characterized by almost invisible chlorotic areas. Later small areas enlarged and turned into light green mosaic with streaks. The contrast betwe-

Fig. 1. Mosaic symptoms of CymMV on *Cymbidium hybrida* leaves

Fig. 2. Necrotic spot symptoms of CymMV on *Cymbidium hybrida* flower

en light and dark areas became more pronounced in older leaves and the symptoms became conspicuous (Fig. 1). Necrotic spots were found on leaves of some cultivars; on flowers they occured rarely but were found on some cultivars, for example, Velmirage (Fig. 2). H. Lawson indicated that flower necrosis was observed in *Cattlea* species for many years and

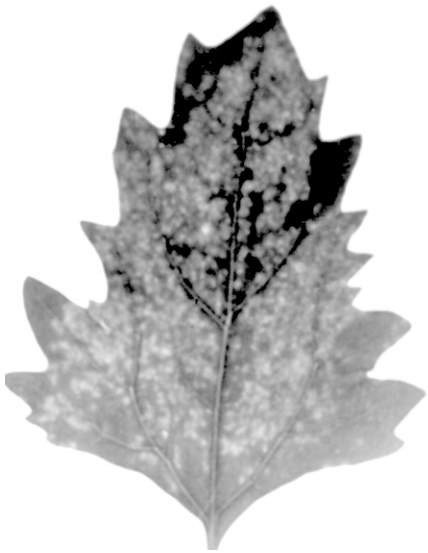

Fig. 3. Local lesions induced by CymMV on inoculated *Chenopodium quinoa* leaf

Fig. 4. Local lesions induced by CymMV on inoculated *Datura stramonium* leaf

was regarded as a symptom of senescence. Later it was shown that these symptoms were induced by CymMV [1].

Virus was experimentally inoculated into test-plants which expressed the following reactions:

Chenopodium amaranticolor – light green local lesions on inoculated leaves;

C. quinoa – rare light yellow local lesions with a necrotic centre about 3 mm in diameter (Fig. 3);

Datura stramonium – differently shaped local lesions on inoculated leaves 14–15 days after inoculation (Fig. 4). This species is a differential test-plant for CymMV and very suitable to separate this virus from others in mixed viral infections which are very common in orchids [14, 15].

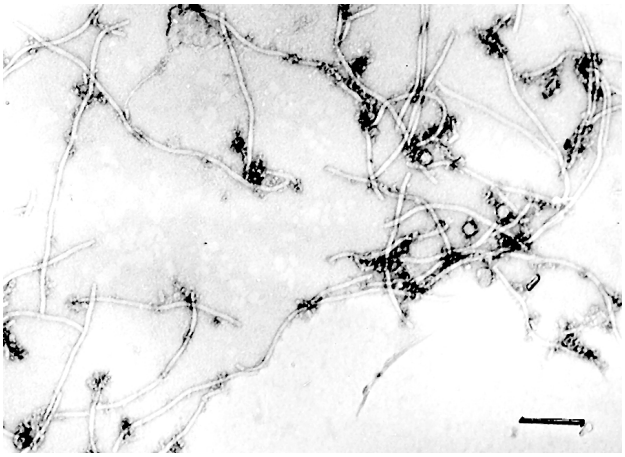

Fig. 5. Particles of CymMV. Bar represents 100 nm

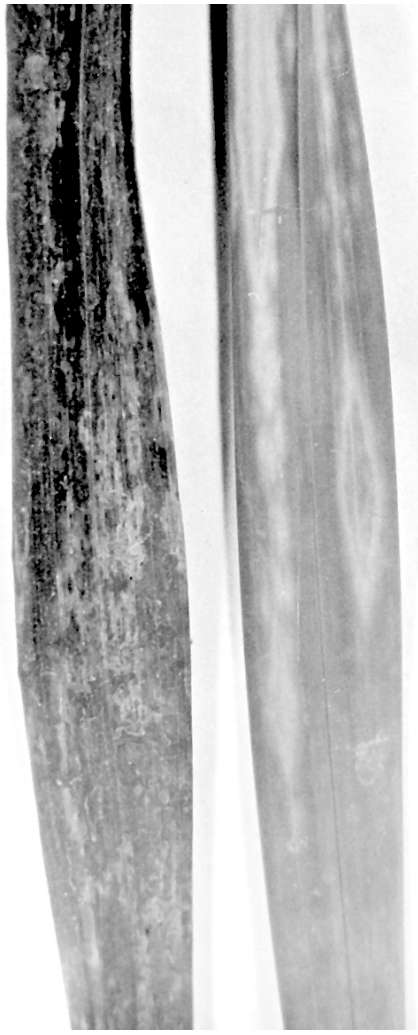

Fig. 6. Mosaic symptoms of ORSV on *Cymbidium hybrida* leaves

Tetragonia expansa – developed small chlorotic local lesions 15–25 days after inoculation.

Cymbidium plants and *D. stramonium* are described as suitable for maintaining CymMV and as a source for purification [15, 18]. In our experiments, *C. quinoa* was used as a source for virus purification and gave good yields. The selected and modified method of purification permitted to obtain a purified CymMV preparation with a yield of 7.5 mg virus from 100 g of infected *C. quinoa* leaf tissue. The purified preparation spectrophotometrically had A_{max} at 260 nm and A_{min} at 240 nm wave length, the $A_{260/280}$ ratio being 1.09. These data are characteristic of purified nucleoproteins [14].

Electron microscopy of negatively stained dip preparations prepared from inoculated test-plants and purified CymMV solution revealed flexuous filamentous particles 480–500 nm in length (Fig. 5). In plant virus descriptions it is indicated that CymMV has filamentous not enveloped particles with a clear modal length of 480 nm, 13 nm wide, axial canal obscure [15].

The purified CymMV preparation was used as an antigen for rabbit immunization. The produced specific antiserum did not react with crude sap of healthy plants. The titre of the produced specific to CymMV diagnostic antisera was 1:2560 in DAS-ELISA test. IgG fraction from antiserum was precipitated and purified.

Odontoglossum ringspot tobamovirus. Infected leaves showed a mosaic consisting of rhombus-shaped light chlorotic blotches (Fig. 6). Black or brown necrotic spots or sunken brownish necrotic spots or streaks were found on older infected leaves. Severe infection leads to a premature death of leaves.

The virus was experimentally inoculated into test-plants and induced the following reactions:

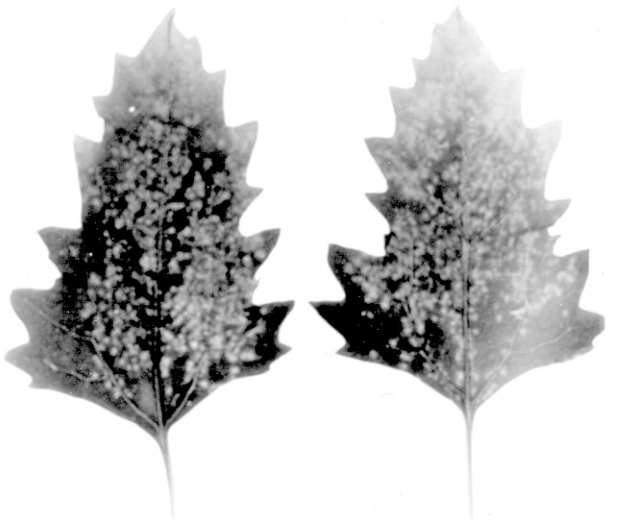

Fig. 7. Local lesions induced by ORSV on inoculated *Chenopodium quinoa* leaves

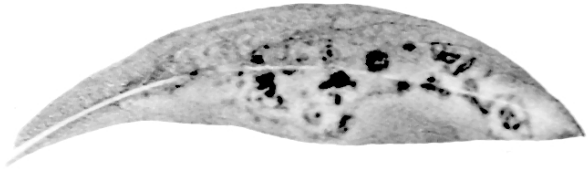

Fig. 8. Necrotic local ring-like lesions induced by ORSV on inoculated *Gomphrena globosa* leaf

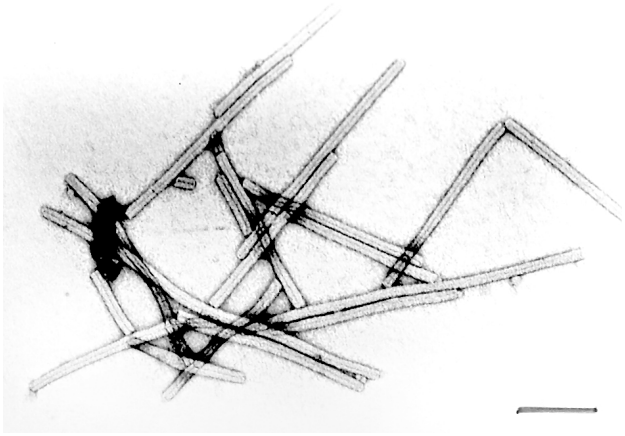

Fig. 9. Particles of ORSV. Bar represents 100 nm

Chenopodium amaranticolor – local necrotic lesions on inoculated leaves;

C. quinoa – chlorotic turning to necrotic local lesions on inoculated leaves (Fig. 7);

Gomphrena globosa – necrotic local ring-like lesions on inoculated leaves (Fig. 8). This species is a differential test-plant for ORSV.

Tetragonia expansa – necrotic ash-colour local lesions on leaves. Later leaves became yellow.

The yield of purified ORSV preparation was 12.4 mg from 100 g of infected *C. quinoa* tissue. The purified preparation spectrophotometrically had A max at 260 nm and A min at 240 nm wave length, the A 260/280 ratio 1.19. These data are characteristic of purified nucleoproteins [13].

Electron microscopy of negatively stained dip preparations prepared from inoculated test-plants and purified ORSV solution revealed rod-shaped particles 290–300 nm in length (Fig. 9).

In plant virus descriptions it is indicated that ORSV has rod-shaped, not enveloped, usually straight particles with a clear modal length of 300 nm, 18 nm wide, axial canal obvious [13].

The purified ORSV preparation was used as an antigen for rabbit immunization. The produced specific antiserum did not react with the crude sap of healthy plants. The titre of the prepared specific diagnostic antisera to ORSV was 1:40960 in DAS-ELISA test. The IgG fraction from antiserum was precipitated and purified. Specific enzyme-labeled antibodies (conjugates) with horseradish peroxidase and inorganic pyrophosphatase of *Escherichia coli* were prepared and estimated. A DAS-ELISA system was prepared with our conjugates. Optimum conditions for the DAS-ELISA test were developed: antigen concentration for adsorbtion on microplates – 10.0 to 3.3 μg/ml IgGs concentration – 10 μg/ml, dilution of conjugates – 1:400–1:800. Our experiments showed the possibility to use an inorganic pyrophosphatase of *Escherichia coli* for labelling IgG instead of horseradish peroxidase or alkaline phosphatase usually used in the detection of plant viruses by ELISA [19]. In our experiments with inorganic pyrophosphatase, the background was very low (A620 < 0.02) – approx. 4 times lower than with horseradish peroxidase. Inorganic pyrophosphatase gave good results and high green colour intensities (A620 > 0.8). Our method permitted a reliable detection of ORSV directly in crude sap from Cymbidium leaves (diluted 1:1000).

CymMV and ORSV are the most prevalent and economically important viruses infecting orchids worldwide [1, 5]. They are stable and easily mechanically transmissible [7]. Control of these two viruses is difficult due to their stability, existence of symptomless hosts, the frequent handling of plants during harvesting, and no known source of genetic resistance. An integrating approach utilizing a virus-free propagation stock, sanitary culture, and genetically engineered orchids resistant to viruses may provide a more complete control of these important virus diseases [20]. To facilitate virus detection, sensitive techniques are developed and continuously improved. Although recently more sensitive techniques for virus detection have been developed [20, 21], DAS-ELISA remains indispensable when screening large numbers of samples.

Received 27 December 2003
Accepted 23 November 2004

References

1. Lawson RH, Hsu HT. Orchid. In: Loebenstein G, Lawson RH, and Brunt AA (eds). Virus and virus-like diseases of bulb and flower crops. Chichester, 1995; 409–20.
2. Turner RR. Am Orch Soc Bull 1981; 50(5): 515–23.
3. ×åðåâ÷åíêî ÒÌ, Êóøíèð ÒÏ. Îðõèäåè â êóëüòóðå. Êèåâ, 1986.
4. Lawson RH, Brannigan M. Virus diseases of orchids. In: Handbook of Orchid Pests and diseases. 1986: 2–49.
5. Zettler FW, Ko NJ, Wisler MS et al. Plant Dis 1990; 74: 621–6.
6. Wisler GC, How to Control Orchid Viruses: the Complete Guidebook. Gainesville, 1989.
7. Eun AJC, Wong SM. Phytopathology 1999; 89: 522–8.
8. Kado CI, Agrawal HO (eds.) Principles and Techniques in Plant Virology. New York, 1972.
9. Noordam D. Identification of Plant Viruses. Methods and Experiments. Wageningen, 1973.
10. Robinson DG, Ehlers U, Herken R. Methods for Electron Microscopy. An Introduction for the Biomedical Sciences. Springer-Verlag, Berlin, 1987.
11. Frowd JA, Tremaine JH. Phytopathology 1977; 67: 43–9.
12. Zaitlin M, Israel HW. CMI/AAB. Description of Plant Viruses 1975; 151: 1–6.
13. Odontoglossum ringspot tobamovirus. In: Brunt AA, Crabtree K, Dallwitz MJ et al (eds.). Plant Viruses

Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. URL http://biology.anu.edu.au/Groups/MES/vide/
14. Koenig R, Lesemann DE. CMI/AAB. Description of plant viruses 1978; 200: 1–5.
15. Cymbidium mosaic potexvirus. In: Brunt AA, Crabtree K, Dallwitz MJ et al (eds.). Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. URL http://biology.anu.edu.au/Groups/MES/vide/
16. Clark MF, Adams AN. J Gen Virol 1977; 34: 475–83.
17. Baykov AA, Kasho VN, Avaeva SM. Analyt Biochem 1988; 171: 271–6.
18. Vejaratpimol R, Channuntapipat C, Pewnim T et al. J Biosc Bioeng 1999; 87: 161–8.
19. Gera A, Lawson RH, Hsu H-T. In: Loebenstein G, Lawson RH, Brunt AA (eds). Virus and Virus-like Diseases of Bulb and Flower Crops. Chichester, 1995: 165–80.
20. Barry K, Hu JS, Kuehnle AR et al. J Phytopathol 1996; 144: 179–86.
21. Hyun Ryu K, Yoon KE, Won Mok Park. J Phytopathol 1995; 643–6.

M. Navalinskienë, J. Raugalas, M. Samuitienë

GËLIØ VIRUSINËS LIGOS 16. ORCHIDËJAS (*CYMBIDIUM* SW.) PAÞEIDÞIANÈIØ VIRUSØ IDENTIFIKACIJA

S a n t r a u k a

Ið Lietuvoje auginamø *Cymbidium* Sw. augalø iðskirti ir nustatyti du virusai: cimbidþiø mozaikos poteksvirusas (*Cymbidium mosaic potexvirus*, CymMV*)* ir *Odontoglossum* þiedið-kosios dëmëtligës tobamovirusas (*Odontoglossum ringspot tobamovirus*, ORSV*)*. Virusai identifikuoti augalø indikatoriø ir elektroninës mikroskopijos metodais. Parinktos metodikos virusams iðgryninti ir paruoðti iðgryninti virusø preparatai (antigenai), kuriø iðeiga buvo: CymMV – 7,5 mg viruso ir ORSV – 12,4 mg viruso ið 100 g pradinës medþiagos. Pagaminti specifiniai diagnostiniai antiserumai turëjo titrus, nustatytus DAS-ELISA metodu: 1: 2560 – CymMV ir 1: 40960 – ORSV. Ið antiserumø iðskirti specifiniai CymMV ir ORSV imunoglobulinai. Pagaminti ORSV IgG konjugatai su krieno peroksidazës ir neorganinës *Escherichia coli* pirofosfatazës fermentais ir palygintos jø panaudojimo DAS-ELISA galimybës. Parinktos optimalios DAS-ELISA sistemos sàlygos. Atskleisti konjugato su neorganine pirofosfataze privalumai.