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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF RED CLOVER NECROTIC MOSAIC VIRUS IN THE HOST PLANTS

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Summary. – Detection of three isolates of red clover necrotic mosaic virus (RCNMV) representing A, B, and C serotypes was experimentally proved in 18 host plant species by enzyme-linked immunosorbent assay (ELISA). In all plant species tested, the homologous serotype reactions showed high selectivity. Individual virus serotypes could be reliably detected in the extracts of infected plants only with the homologous IgG fraction. Group specific detection of RCNMV without serotype determination was possible using the mixture of IgG directed to all virus serotypes occurring in the region of investigation. Intensity of positive reaction of optimally diluted IgG with the extracts from infected plants differed markedly from that of negative reaction and from the reaction background. The latter depended on the quality of serum used for the IgG preparation. For detection of small amounts of RCNMV, virus infectivity test on indicator plants was more sensitive than ELISA.

Key words: red clover necrotic mosaic virus; virus detection; ELISA

Introduction

ELISA has become more and more widely used also for detection and identification of forage legume viruses (McLaughlin and Scott, 1986). Under optimal conditions it can be used for detection as well as for identification of viruses with a low or practically no antigenic variability. More complicated is simultaneous detection and identification of serologically closely related viruses, such as members of the *Comovirus* group (Gallo and Musil, 1988) or viruses with a great serological variability, some serotypes of which react selectively in ELISA (Koenig, 1978; Van Regenmortel, 1982). Selectivity of ELISA is also typical for the serotypes of RCNMV belonging to the *Dianthovirus* group (Hiruki *et al.*, 1984). It is caused mainly by different quantitative representation of the used IgG directed to individual RCNMV serotypes of antibody groups taking part in

the serotype-specific homologous reaction or, on the contrary,in reaction with heterologous antigen (Musil and Gallo, 1990).

With regard to the above mentioned facts, we examined experimentally the possibility of the optimal use of direct ELISA for detection and identification of three RCNMV serotypes (A, B, and C) in different host plants. This paper summarizes the results of comparative experiments with three RCNMV isolates, representing the A, B, and C serotypes spread in Czechoslovakia (Musil and Gallo, 1982; Musil *et al.*, 1982).

Materials and Methods

Viruses. Three RCNMV isolates, namely TpM_{34} (serotype A), TpM_{48} (serotype B), and isolate No.6 (serotype C), designated further in numbers only, were used. To determine the titres of virus-specific antibodies in original antisera and corresponding IgG fractions, we prepared purified suspensions of individual virus isolates (Musil and Gallo, 1990); they were used for determination of optimal IgG dilutions in ELISA.

Individual serotypes of RCNMV or their mixtures were determined in the extracts from 18 plant species which were infected in parallel with individual RCNMV isolates or their mixtures; in addition, extracts from noninfected plants and from those infected with a serologically unrelated virus, alphaalpha mosaic virus (AMV), were also tested. Individual RCNMV isolates, their mixtures, or AMV were inoculated to experimental plants as described (Musil *et al.*, 1981). Virus-inoculated and non-inoculated plants were grown in an insect-free greenhouse. Samples of the leaves to which the virus was applied were collected 7 days post-inoculation; on day 28 the samples were collected from leaves which were grown up after virus inoculation, i. e. from non-inoculated leaves. In the case of apparent local or systemic plant infection, the samples were collected from the leaves with developed signs of virus infection. From non-inoculated plants, the samples were collected from the leaves of the same age as from those of virus-inoculated plants.

Preparation of the antigen extracts. Leave samples (mass 0.4 g) were ground in 4 ml sampling buffer and the homogenate was divided into 3 tubes, frozen and stored at -20 ^{0}C. The homogenates were thawed just before testing (24 hr to several weeks or even months after preparation); the remnants of plant tissues were removed by low-speed centrifugation (15 min at 3–4,000 x g) and the cleared extracts were used for ELISA either undiluted or diluted in the sampling buffer. In the same buffer we diluted the purified suspensions of individual RCNMV isolates.

Immunoglobulin (IgG) fractions against the RCNMV isolates were prepared from hyperimmune rabbit antisera to 6 RCNMV isolates No. 34, 48, and 6 (Musil and Gallo, 1990). Anti-34, anti-48, and anti-6 IgG fractions were adjusted to the same protein concentration (1 mg/ml); a part of IgG was used for coating of the wells and the rest was conjugated with alkaline phosphatase (type V11, SIGMA; 1,000 U per ml). Both the coating IgG ant the conjugates were used either separately or in mixture (for dilution buffer see Clark and Adams, 1977).

ELISA. The double antibody sandwich was used throughout as described by Clark and Adams (1977) on domestic microtitration plates (KOH-I-NOR, Dalečín, Czechoslovakia). Wells were coated with 100 μl of anti-IgG or their mixture, diluted according to the purpose of the experiment or according to the titre of virus-specific antibody. Then 100 μl of antigen was added to the wells, washed and thereafter the conjugate or their mixture was added.

Test of virus infectivity on indicator plants. The extracts from the virus-inoculated plants were diluted from 10^{-1} to 10^{-5} and the virus infectivity was tested on the primary bean leaves (*Phaseolus vulgaris*, cv. Perlička), no parallel with testing of the antigen in extracts. Serial dilutions of the extracts were inoculated mechanically onto the primary leaves of 14-days-old bean plants which were then grown in the greenhouse. Virus infection was evaluated visually, based on the presence of necrotic lesions or vein necroses on the inoculated leaves (Musil *et al.*, 1981).

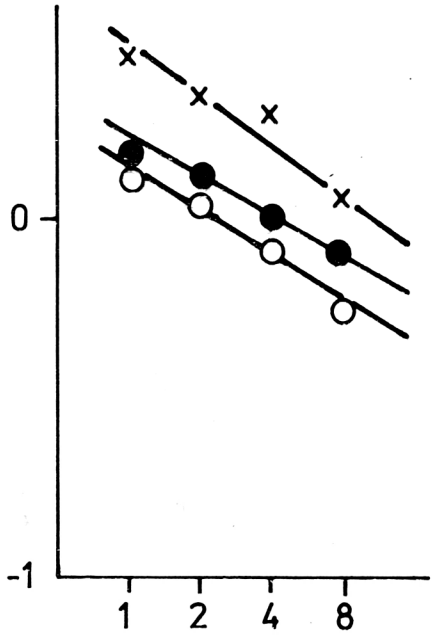

Fig. 1
Intensity of ELISA reaction using gradually diluted conjugates 34 (x), 48 (●), and 6 (○) with the homologous antigen (purified virus suspensions diluted 1:1000); dilution of coating IgG 1:1000. Abscissa: conjugate dilution ($x10^{-3}$); ordinate: absorbance at 410 nm (log_{10}).

Results

Precipitating antibodies in rabbit antisera and in their IgG fractions

Antisera from which the IgG fraction was extracted contained precipitating antibodies to homologous antigen in the titre of 256 (antiserum 48) and 512 (antisera 6 and 34), respectively. In the IgG fractions were adjusted to the same concentration of 1 mg/ml, the titres of precipitating antibodies in the homologous reaction were 128 (IgG anti-34) and 64 (IgG anti-6 and anti-48), respectively. Apart from these serotype-specific antibodies also low titres of heterologous antibodies were found: in antiserum 6, the titre of antibody to antigen 48 was 16, but no antibody to antigen No. 34 was detected; in antiserum 34 the titre of antibody to antigen 6 was 16, and to antigen No. 48 it was 8; in antiserum 48, the highest heterologous antibody titres were recorded, namely 64 to antigen No. 6 and 128 to antigen No. 34. However, in the IgG fractions adjusted to the concentration of 1mg/ml, the ring precipitation revealed no heterologous antibody activity; in anti-6 and anti-34 IgG preparations a low titre of 16 to antigen 34 was found in the anti-48 IgG preparation only.

Reactivity of IgG 6, 34, and 48 with homologous and heterologous RCNMV antigens

Serotype-specific antibodies in individual IgG fractions (within the range of dilutions from 1:1,000 to 1:8,000) with homologous antigens yielded noticeable

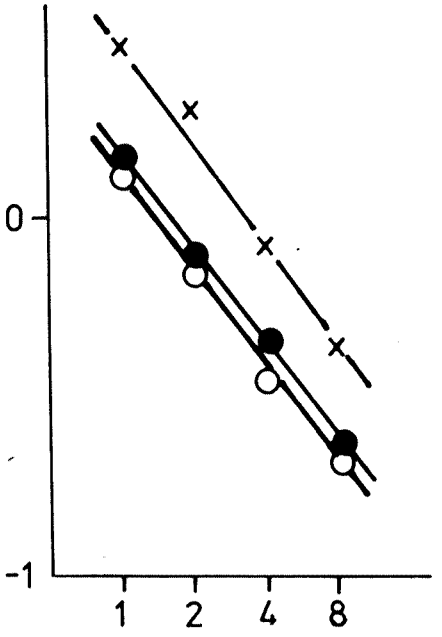

Fig. 2
Intensity of ELISA reaction using gradually diluted purified virus suspensions 34 (x), 48 (●), and 6 (○) against the homologous conjugate and coating IgG diluted 1:1000
Abscissa: conjugate dilution ($x10^{-3}$); ordinate: absorbance at 410 nm (log_{10}).

absorbance values (Fig. 1). IgG anti-34 reacted with homologous antigen almost at two-fold higher intensity than IgG anti-6 or IgG anti-48, respectively. The heterologous antigens (purified virus suspensions, diluted 1:1,000) reacted with the individual IgG fractions (diluted 1:1,000) only at a low intensity, the measured absorbance values being lower than 0.10. The only exception was the reactivity of IgG 48 with antigen 34 with the absorbance values around 0.20. The intensity of homologous reactions in ELISA was directly proportional to the conjugate dilution and depended, though not fully proportionally, on the concentration (dilution) of purified suspensions of the individual RCNMV isolates used (Fig. 2).

Similarly as with purified viral suspensions, the IgG fractions reacted in ELISA also with the extracts from bean plants infected with individual RCNMV isolates (Table 1). The conjugate 34 reacted at two-fold higher intensity with the extract from the plant infected with the homologous strain than the conjugates 6 and 48. When binding the antigen to antibody of the coating IgG fraction (diluted 1:1,000 and 1:2,000, respectively), the individual conjugates reacted with the extracts containing homologous RCNMV antigen in a sufficiently intensive positive reaction (the absorbance values were higher than 1.00). The intensity of final reaction depended on dilutions of both coating and conjugated IgG. For serial detection of individual RCNMV isolates in the extracts from different plant species as suitable appeared dilution 1:1,000 for IgG anti-6 and IgG anti-48 or dilution 1:2,000 for IgG anti-34, when used either

for coating and/or as conjugates. These dilutions eliminated sufficiently the nonspecific reactions with normal plant protein antigens present in the plant extracts. Similar low intensity of reactions was noticed with the abovementioned IgG dilutions and with the extracts from plants infected with heterologous RCNMV isolates, the absorbance values corresponding to those found with the extracts from healthy (non-infected) plants.

Of the IgG fractions used most specifically reacted the IgG 48, whereas IgG anti-34 reacted with the normal plant antigens at a substantially higher intensity, e. g. in dilution 1:1,000 of the conjugated IgG in combination with the extracts from healthy plants the absorbance reached even 0.50. Similar reactivity of the same conjugate was observed also with the extracts from plants

Table 1. ELISA of RCNMV in the extracts from the leaves of bean *(Phaseolus vulgaris*, cv. Perlička) infected with 34, 48, and 6 RCNMV isolates as related to concentrations of coating IgG and conjugates

IgG	Dilution of coating IgG	Dilution of the conjugate	Extracts from plants			
			infected with isolate			uninfected
			34	48	6	
34	1:1.000	1:1000	3.00*	0.52	0.49	0.50
		1:2000	3.00	0.18	0.16	0.18
		1:4000	1.65	0.13	0.13	0.11
	1:2.000	1:2000	2.84	0.15	0.16	0.14
		1:4000	1.14	0.12	0.11	0.10
		1:8000	0.52	0.13	0.12	0.07
	1:4.000	1:2000	1.09	0.03	0.07	0.07
		1:4000	0.53	0.02	0.03	0.02
		1:8000	0.27	0.03	0.03	0.02
48	1:1.000	1:1000	0.13	1.84	0.11	0.03
		1:2000	0.01	1.17	0.01	0.02
		1:4000	0.03	0.57	0.02	0.02
	1:2.000	1:1000	0.02	1.63	0.02	0.02
		1:2000	0.01	0.86	0.01	0.01
		1:4000	0.01	0.36	0.02	0.02
6	1:1.000	1:1000	0.02	0.10	1.54	0.02
		1:2000	0.01	0.04	0.83	0.01
		1:4000	0.01	0.03	0.41	0.01
	1:2.000	1:1000	0.04	0.12	1.13	0.02
		1:2000	0.01	0.05	0.78	0.02
		1:4000	0.01	0.02	0.38	0.01

* absorbance at 410 nm

infected with a heterologous RCNMV isolate or with an unrelated virus (e. g. AMV). On the contrary, the conjugated IgG anti-48 in dilution 1:1,000 reacted with the extracts from healthy bean plants at lower intensity than with the extracts from plants infected with a heterologous RCNMV isolate. In the latter case, the reaction of virus-specific antibodies directed to heterologous antigen and the virus-nonspecific reaction were additive. In a similar way, though not so markedly, reacted the conjugated IgG anti-6 (dilution 1:1,000) but only with the extract from the plants infected with the 48 RCNMV isolate; the intensity of reaction with the extract from the plants infected with 34 isolate was similar to that with the extract from healthy (non-infected) bean plants. Summing up the intensity of reactions of conjugated IgG diluted 1:1,000 with heterologous antigen reached only low absorbance values (around 0.10). These values could not be sufficiently differentiated from those of negative virus-nonspecific reactions, namely when testing the extracts from different plant species containing unequal concentrations of viral antigen. Conjugated IgG diluted 1:2,000 (IgG anti-6 and IgG anti-48) and 1:4,000 (IgG 34), respectively, reacted with the plant extracts containing heterologous antigen at intensity not surpassing that of the reaction with the extracts from healthy plants (Table 1).

In the extracts from the bean plant prepared separately from the virus-inoculated leaves (local infection) and from the leaves grown up after virus inoculation, ELISA in either case revealed the lower multiplication of RCNMV isolate 6 than isolates 34 and 48, at the same time in the individual isolates no substantial difference was found between the local and systemic infection (Table 2).

In the pea and broad bean plants viral antigen was detected only within the extracts from virus-inoculated leaves, but not in leaves grown after virus inoculation. In the inoculated leaves of the pea, individual RCNMV isolates multiplied in a similar way as far as the appearance and concentration of the viral antigen are concerned. In the inoculated leaves of the broad bean isolate No. 34 multiplied more rapidly and to higher concentration than isolates 48 and 6; the isolates 6 yielded markedly less antigen during the same period of time than the two RCNMV isolates. The intensity of negative reactions of individual conjugated IgG with the extracts from the non-inoculated plant leaves and with those from locally infected plants using homologous or heterologous RCNMV isolates did not differ distinctly from the intensity of reaction with the extracts from leaves of healthy (noninfected) plants and from plants infected with a serologically unrelated virus (Table 2).

Detection of RCNMV by ELISA and infectivity test

In gradually ten-fold diluted extracts from inoculated and noninoculated leaves of bean, pea, and *Trigonella coerula*, the viral antigen was detected by ELISA in dilutions of 10^{-2} to 10^{-3} (pea) and 10^{-3} to 10^{-4} (bean and *Trigonella coerulea*) extracts, respectively. Infectivity tests demonstrated the virus in ten times higher dilutions (Table 3). This difference reflects the different evaluation of reaction positivity; while low absorbance values in ELISA are difficult to assess because of background reactions, the infectivity test on indicator plants

Table 2. ELISA of RCNMV in the extracts from pea (*Pisum sativum, cv. Juran*), broad bean (*Faba vulgaris*, cv. Inovec), and bean (*Phaseolus vulgaris*, cv. Perlička) infected with 34, 48, and 6 RCNMV isolates (coating dilution 1:2000 for IgG anti-6 and IgG anti-48, and dilution 1:4000 for IgG anti-34; dilution of each conjugate 1:2000)

Host plant species	Form of infection	Extract from the leaves	IgG fraction		
			anti-34	anti-48	anti-6
Pisum sativum	None	ni	0.03	0.03	0.02
	Isolate No.34	i	0.83	0.03	0.03
		n	0.06	0.01	0.03
	Isolate No.48	i	0.05	0.55	0.03
		n	0.04	0.02	0.04
	Isolate No.6	i	0.03	0.03	0.44
		n	0.06	0.02	0.02
	AMV-system	n	0.04	0.01	0.03
Faba vulgaris	None	ni	0.05	0.01	0.02
	Isolate No.34	i	1.09	0.01	0.02
		n	0.04	0.01	0.02
	Isolate No.48	i	0.05	0.51	0.02
		n	0.04	0.03	0.02
	Isolate No. 6	i	0.05	0.02	0.28
		n	0.06	0.02	0.02
Phaseolus vulgaris	None	ni	0.04	0.01	0.01
	Isolate No.34	i	1.09	0.03	0.01
		n	1.03	0.01	0.02
	Isolate No.48	i	0.02	0.93	0.03
		n	0.03	0.72	0.01
	Isolate No.6	i	0.03	0.02	0.49
		n	0.02	0.02	0.35

ni - leaves from non-infected plants
i - leaves to which the virus was inoculated 3-7 days ago
n - non-inoculated leaves or grown up after the virus inoculation

allows an unequivocal demonstration of minimal virus amounts.

ELISA of RCNMV serotypes in different host plants

The extracts from all species of host plants under study infected by individual RCNMV isolates reacted in ELISA only with homologous IgG, the positive reaction being clearly distinct from the negative reaction and the background reaction, respectively. The absorbance values of positive reactions were usually 3.00 or two times higher than the absorbance values of negative reactions (Table 4). Different proportion of antibodies against normal plant anti-

gens in the IgG preparations appeared in reactions with the extracts from from healthy plants or from those infected with the heterologous RCNMV isolate and the serologically unrelated virus. The intensity of these reactions with IgG 34 reached the absorbance values of 0.10 to 0.20 and in some cases even of 0.30 (the extracts from *Medicago lupulina, Melilotus albus,* and *Vicia* sp.). Negative reactions with IgG anti-6 were usually of lower intensity (the absorbance values from 0.10 to 0.25) and those with IgG anti-48 reached 0.04 to 0.17 absorbance values.

Individual serotypes were reliably detected also in the extracts from the plants infected simultaneously with two or even three serologically different RCNMV isolates. The intensity of positive reactions of individual IgG with corresponding antigen (the homologous antigen present in the extracts from the plants infected with a complex of RCNMV serotypes) differed only slightly

Table 3. Detection of RCNMV isolates from three host plant species by ELISA and virus infectivity test on the indicator plant *Phaseolus vulgaris*, cv. Perlička

Host plant species	RCNMV isolate	Form of infection	End-point dilution of the virus or viral antigen as detected by	
			ELISA (absorbance)	Infectivity test
Phaseolus vulgaris	34	li	10^{-4} (0.35)	10^{-5}
		si	10^{-4} (0.25)	10^{-5}
	48	li	10^{-4} (0.35)	10^{-5}
		si	10^{-4} (0.25)	10^{-5}
	6	li	10^{-4} (0.27)	10^{-5}
		si	10^{-3} (0.19)	10^{-5}
Pisum sativum	34	li	10^{-3} (0.10)	10^{-4} to 10^{-5}
		si	0 (0.06)	0
	48	li	10^{-2} (0.35)	10^{-3}
		si	0 (0.07)	0
	6	li	10^{-2} (0.34)	10^{-3}
		si	0 (0.06)	0
Trigonella coerulea	34	li	10^{-4} (0.30)	10^{-4}
		si	10^{-4} (0.31)	10^{-5}
	48	li	10^{-3} (0.13)	10^{-3}
		si	10^{-3} (0.18)	10^{-3}
	6	li	10^{-3} (0.23)	10^{-5}
		si	10^{-3} (0.34)	10^{-5}

li - local infection (on the virus-inoculated leaves), si - systemic infection, 0 - infection changes not detected

Table 4. ELISA of 34, 48, and 6 RCNMV isolates in the extracts from different host plant species; individual or mixed coating IgGs were diluted 1:1000 (IgG anti-6 and anti-48) or 1:2000 (IgG anti-34)

Host plant species	IgG anti-34			IgG anti-48			IgG anti-6			IgG mixture		
	Ag34	Ag48	Ag6	Ag34	Ag48	Ag6	Ag34	Ag48	Ag6	Ag34	Ag48	Ag6
Anthylis vulneraria	3.00	0.15	0.22	0.08	3.00	0.06	0.16	0.09	3.00	3.00	3.00	3.00
Faba vulgaris	0.94	0.11	0.15	0.16	1.88	0.11	0.20	0.10	1.50	0.90	1.48	1.28
Lens culinaris	2.44	0.30	0.21	0.10	3.00	0.08	0.16	0.17	3.00	3.00	3.00	3.00
Medicago lupulina	3.00	0.31	0.33	0.08	1.64	0.07	0.15	0.13	3.00	3.00	1.84	3.00
Medicago sativa	0.24	0.21	0.22	0.07	0.08	0.07	0.12	0.14	0.13	0.19	0.23	0.24
Melilotus albus	3.00	0.35	0.31	0.09	3.00	0.09	0.16	0.16	0.85	3.00	3.00	0.92
Melilotus officinalis	3.00	0.22	0.26	0.09	1.36	0.09	0.21	0.15	3.00	3.00	1.60	3.00
Nicotiana megalosiphon	3.00	0.10	0.13	0.06	1.59	0.05	0.07	0.08	3.00	3.00	1.76	3.00
Trifolium alexandrinum	0.34	0.30	0.12	0.06	0.57	0.06	0.21	0.19	0.09	0.432	0.61	0.21
T. campestris	3.00	0.26	0.14	0.09	3.00	0.06	0.13	0.13	2.43	3.00	3.00	2.62
T. incarnatum	1.00	0.22	0.23	0.08	2.14	0.10	0.13	0.16	3.00	1.20	2.25	3.00
T. pratense	0.83	0.25	0.19	0.07	0.64	0.08	0.16	0.20	1.40	0.78	0.72	1.23
Trigonella coerulea	3.00	0.28	0.15	0.07	1.12	0.06	0.15	0.12	1.22	3.00	1.32	1.12
Trigonella foenum-graecum	3.00	0.23	0.14	0.07	2.23	0.07	0.16	0.16	0.14	3.00	2.42	0.28
Vicia pannonica	3.00	0.25	0.31	0.17	1.41	0.10	0.19	0.26	3.00	3.00	1.67	3.00
Vicia sativa	3.00	0.22	0.34	0.12	3.00	0.11	0.23	0.19	3.00	3.00	3.00	3.00
Vicia villosa	0.89	0.31	0.32	0.09	1.82	0.10	0.24	0.26	3.00	1.04	2.15	3.00

Ag – viral antigen

from that with the extracts from the plants infected with a single RCNMV isolate. Similar results were also obtained when testing individual RCNMV serotypes in the extracts from the plants infected with the single or mixed RCNMV isolates by the use of the individual or mixed IgG preparations. Simultaneous detection of antigenically different isolates was manifested by increased intensity of the reaction which was not always equal to the sum of expected serotype reactions. In most cases, the reactions of mixed antibodies with a mixture of two or three RCNMV serotypes present in the extracts displayed the absorbance value of 3.00. Simultaneous detection of different serotypes however, required not only the use of a conjugate mixture, but also a mixture for coating to ensure the binding of each antigen. When using a single serotype anti-IgG for coating, no binding of heterologous antigen would take place, which results in the inability to detect this antigen by corresponding conjugated IgG included in the conjugate mixture.

Discussion

As follows from the results of abovementioned tests, in ELISA of three RCNMV serotypes in the host plants, the optimally diluted IgG preparations (IgG anti-6, IgG anti-34, and IgG anti-48) gave a marked positive reaction with homologous antigens only. Intensity of such selective serotype reactions differs distinctly from that of negative and background reactions. The obtained absorbance values of positive reactions in individual plant species express the quantitative proportion of individual RCNMV serotypes in the extracts tested. Differences in the intensity of ELISA reactions between individual plant species and also between the isolates studied in one plant species reflect the different susceptibility of individual plant hosts to RCNMV and its serotypes, respectively.

In ELISA the individual IgG cannot detect the heterologous antigen in the infected plant extracts, not even at optimal dilution (Musil and Gallo, 1990). Heterologous reaction can be proved with purified suspensions of individual RCNMV isolates and using less diluted IgG. However, even in such case, the intensity of heterologous reactions remains, of course, lower than that of the homologous reaction (Musil and Gallo, 1990).

Infection of the host plants with individual or mixed (in a complex) RCNMV serotypes in ELISA can be proved either by the parallel testing of the plant extracts with IgG preparations directed to individual RCNMV serotypes occurring in the area under study or by the use of a mixture of serologically different IgG preparations, corresponding to the serotype representation of RCNMV in the given locality or territory.

For simple detection of RCNMV in host plants without serotype identification, we found suitable a mixture of IgG preparations directed to the serotypes A, B, and C so far found in Czechoslovakia (Musil *et al.*, 1982). The use of sero-

typically different IgG is purposeful namely when investigating the RCNMV occurrence in the host plants in different localities or when examining the degree of infestation of the host plant by the virus. The mere detection of RCNMV without its further serotype determinantion by a mixture of serotypically different IgG saves work and material. Proving the presence of RCNMV as viral species is sufficient for confirming whether the virus participated in the mosaic disease of plants in given locality or in a wider region, i. e. to find out the virus occurrence and distribution. Similarly, to determine the participation of RCNMV in the mosaic disease of the plants in the fields as well as to determine experimentally susceptibility or non-susceptibility of cultivars of some host plants to RCNMV, its detection as viral species only is sufficient. However, in more detail studies of the resistance of individual host plants and their cultivars to RCNMV, determination of the individual RCNMV serotypes is necessary. Then, in such a type of work it is more suitable to investigate the field resistance of spontaneously infected plants and to determine the degree of their resistance at experimental infection in the greenhouse, using ELISA with individual RCNMV isolates even when the plants were infected by a complex of RCNMV serotypes. Similarly, in studies on the pathogenesis of RCNMV infection or in the investigation of antigenic properties of different RCNMV isolates, the use of selective ELISA reactions is purposeful for closer characterization of the isolates.

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