Rapid Detection and Titer Evaluation of Viruses in Pepper by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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The enzyme-linked immunosorbent assay (ELISA) enabled a rapid and accurate detection of potato virus Y (PVY), cucumber mosaic virus (CMV), alfalfa mosaic virus, and tobacco mosaic virus in pepper. The sensitivity of this method made it possible to detect CMV or PVY: as early as 4 and 6 days after inoculation, respectively; directly, in leaf disks without prior homogenization; and in mixed batch samples of infected and healthy plants at a ratio of 1:50. The latter application could enable efficient screening of pepper plant nurseries or virus detection in the field. ELISA also could be used to detect PVY in potato (leaf and tuber), tomato, tobacco, *Physalis floridana*, and *Nicotiana glutinosa*. Cucumber mosaic virus could be detected in all the above hosts, except potato, as well as in cucumber, celery, melon, squash, and *Datura stramonium*. The relative titer of CMV or PVY in peppers could be evaluated by ELISA, which may become useful in breeding peppers by providing a quantitative evaluation of virus resistance.

Peppers (*Capsicum annum* L.) commonly are infected by many viruses (17), among which potato virus Y (PVY) and cucumber mosaic virus (CMV) are probably the most important economically. Both PVY and CMV comprise a large number of strains, have a wide range of hosts, and are spread by aphids in a nonpersistent manner (17). Various cultural practices have been developed for reducing the incidence of viruses in peppers (2,8,9), but since these are as yet partial solutions, breeding remains the principal means of combating virus in peppers. Sources of PVY- and CMV-resistances have been found and introduced into different pepper cultivars (4,5,11), but most of these resistances are subject to breakdown due to the appearance of other viral strains (4,13,14). Breeding for virus resistance in pepper is still done by evaluating the severity of the symptoms visually which, besides many other limitations (6), enables selection only if differences are marked, usually the result of monogenic resistance. Quantifying resistance by assessment of virus titer (7,16) can improve selection, enabling the utilization of polygenic resistances. However, assessment of virus titer(s) seems impractical in pepper because of the difficulty in mechanical transmission from this plant to a local lesion host, probably due to virus inhibitors (10) or as a result of low virus titer (3). The present work was undertaken to find a rapid method for qualitative and quantitative assessment of viruses, especially CMV and PVY, in pepper.

MATERIALS AND METHODS

Virus assessment was done on both naturally and artificially infected plants. Experimental plants were grown in an insect-proof greenhouse and mechanically inoculated at a young stage (first two true leaves fully developed) with virus from cultures routinely maintained in our laboratory. Plants that received more than one virus were inoculated at intervals of 1–2 days. The experimental pepper seedlings used were of a susceptible local cultivar, Zahov Naharia. In naturally infected peppers, virus identity was established by grafting test plants, determining typical host ranges, and occasionally by electron microscopy observations. No reliable results could be obtained by testing sap of these plants with the precipitin and agar double diffusion methods.

Enzyme-linked immunosorbent assay (ELISA) tests were done as described by Clark and Adams (1). Leaves (5 mg) or leaf disks (6 mm in diameter) were homogenized in phosphate-buffered saline solution containing 0.05% Tween-20 and 2% polyvinylpyrrolidone (PBS-buffer). Two hundred microliters of these extracts were added to wells of a microplate (Linbro Scientific Co., Hamden, CT 06511) after the wells had been coated with purified γ-globulin ($A_{280\ nm}$ = 1.4) diluted in a "coating buffer" (1). In some experiments intact leaf disks (6 mm diameter) were directly immersed in the plate wells without homogenization. After an incubation period, enzyme-labeled γ-globulin (conjugated antisera) was added; this triggers the color reaction with the substrate: P-nitrophenyl phosphate. Color intensity was measured at $A_{405\ nm}$ with a colorimeter.

Resistance will be defined according to Schafer (16) correlating it with lower virus titer. For resistance evaluation, at least 10 pepper seedlings from each line tested were grown in an insect-proof greenhouse and were mechanically inoculated at the two true leaf stage. At the same time 10 similar seedlings from each line were rubbed with water. The visual evaluations and ELISA-tests were made by comparison with these controls.

RESULTS

In artificially or field-infected peppers the viruses PVY, CMV, alfalfa mosaic virus (AMV), and tobacco mosaic virus (TMV) could be detected by ELISA with high accuracy in a 2-day process. These results were obtained several times with 3 different sources of antisera and with plants of different ages.

Potato virus Y and CMV (AMV and TMV were not tested) could also be detected in pepper fruits and very old pepper leaves. Potato virus Y could also be easily detected in infected potato (leaf and tuber), tomato, tobacco, *Physalis floridana*, and *Nicotiana glutinosa*. Cucumber mosaic virus could be also detected in the above hosts, except potato, as well as in cucumber, celery, melon, squash, and *Datura stramonium*.

It was also possible to detect PVY and CMV in intact leaf-disks which were directly immersed in the PBS-buffer without prior homogenization. The mean A_{405} obtained by ELISA using single disks from PVY and CMV-infected plants were 0.369 and 0.772, respectively, as compared to $A_{405\ nm}$ of 0.024 obtained with single disks from healthy peppers. However, for quantitative assessment this method does not seem suitable because no correlation was found between the number of disks and the absorbance obtained.

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In the field, peppers may be infected with several viruses. If ELISA is to be used for diagnostic purposes it would be desirable to ensure that the occurrence of one virus does not interfere with the detection of the others. Pepper seedlings were infected with PVY, CMV, AMV, and TMV in all possible combinations and then tested by ELISA. In three independent tests no interference between the different viruses could be observed, and accurate results were obtained according to the antiserum used.

Pepper seedlings were grown under controlled conditions (22 C, 2,500 lux, and a 16-hr photoperiod) and inoculated with CMV and PVY on the first two true leaves; controls were similarly rubbed with water. The occurrence of CMV and PVY was tested by ELISA in the tip (uninoculated) leaves at different intervals after inoculation. The results (Fig. 1,2) indicate that CMV and PVY could be detected as early as 4 and 6 days after inoculation, respectively; symptoms of both viruses appeared in these leaves 3–4 days later.

In three other experiments, we tested the possibility of detecting virus in batches of mixed samples from healthy and diseased plants. One leaf disk from a PVY- or CMV-infected pepper plant was homogenized, with a varying number of disks from healthy peppers in a standard volume of 5 ml PBS-buffer, and tested by ELISA. The results (Fig. 3) indicate that CMV could be detected in a mixture containing one infected disk per 100 healthy disks and PVY could be detected in a 1:50 mixture.

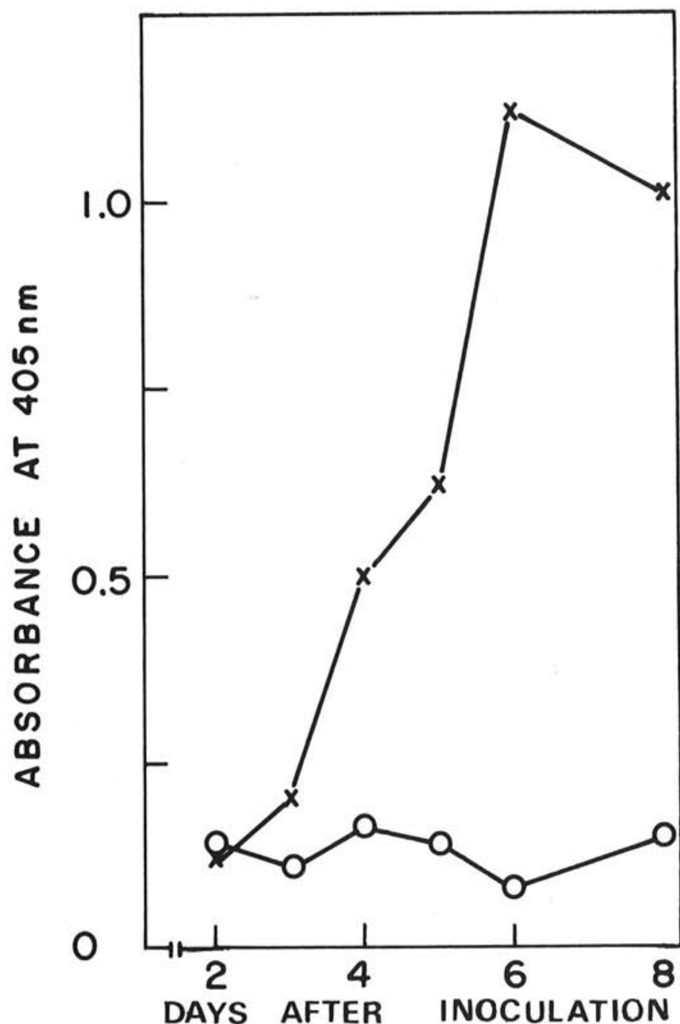

Fig. 1. Absorbance at 405 nm obtained by enzyme-linked immunosorbent assay of extracts (1:100, w/v) from pepper leaves mechanically inoculated with cucumber mosaic virus (X——X) or similarly rubbed with water (o——o). The results are the means of three experiments (five plants per experiment) in which the plants were kept at 22 C, 2,500 lux, and a photoperiod of 16 hr. The dilution of the γ-globulin in the coating buffer was 1:1,000 and that of the conjugated antiserum was 1:2,000.

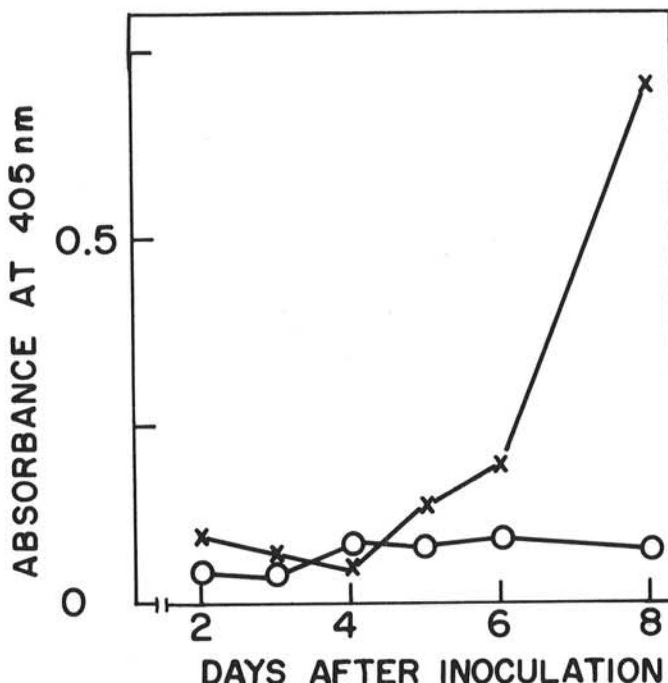

Fig. 2. Absorbance at 405 nm obtained by enzyme-linked immunosorbent assay of extracts (1:100, w/v) from pepper leaves mechanically inoculated with potato virus Y (X——X) or similarly rubbed with water (o——o). The results are the means of three experiments (five plants per experiment) in which the plants were kept at 22 C, 2,500 lux, and a photoperiod of 16 hr. The dilution of the γ-globulin in the coating buffer was 1:100 and that of the conjugated antiserum was 1:200.

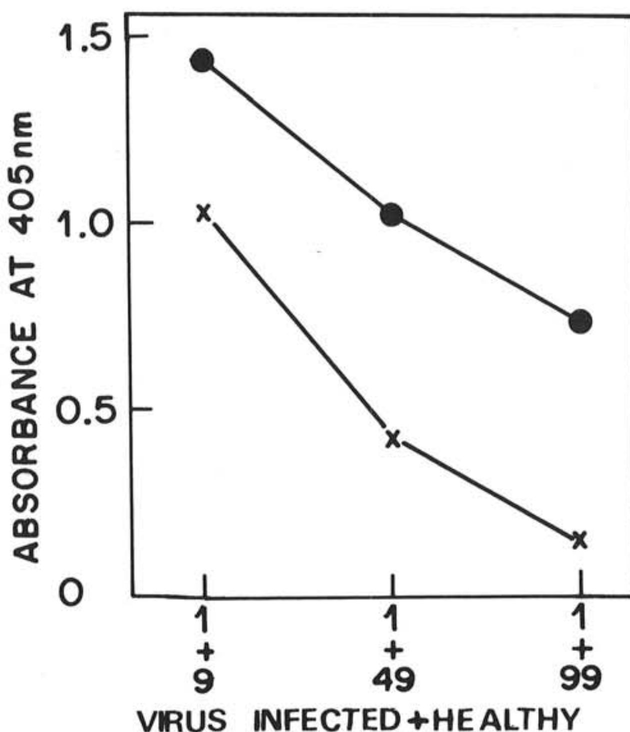

Fig. 3. Absorbance at 405 nm obtained by enzyme-linked immunosorbent assay of extracts from mixed batches of disks from healthy and cucumber mosaic virus (CMV)-infected (•——•) or healthy and potato virus Y-infected (X——X) pepper leaves. A single disk from an infected plant was mixed with varying numbers of disks from healthy plants, and extracts were prepared from the mixture. The results are the means of two experiments after correction for the absorbance obtained with extracts from the same number of healthy disks. The weight of one disk was 5 mg. The dilutions for the γ-globulin were 1:100 for PVY and 1:1,000 for CMV and those of the conjugated antisera were 1:200 and 1:2,000, respectively.

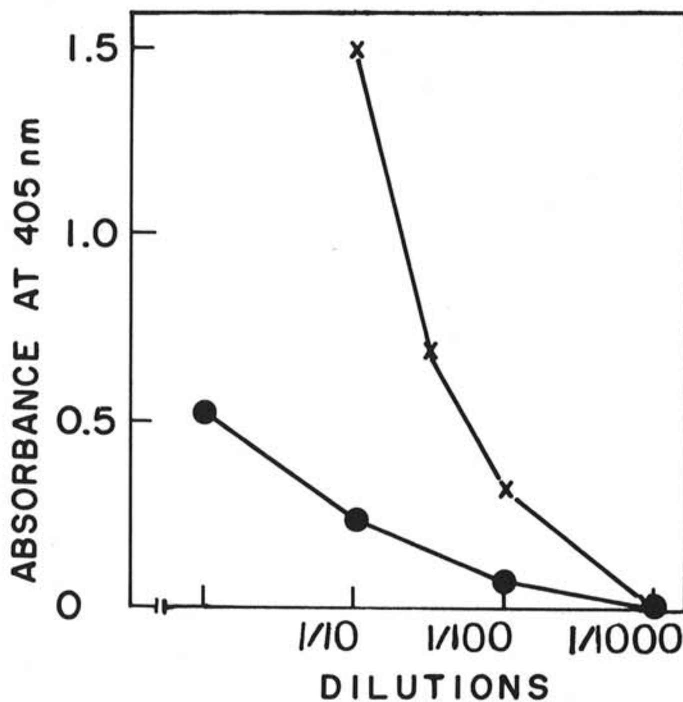

Fig. 4. Absorbance at 405 nm obtained by enzyme-linked immunosorbent assay of dilutions of crude extracts from pepper leaf tissue (•——•) and from suspensions of purified cucumber mosaic virus (×——×). The results are the mean of two experiments. The crude CMV extract (1:100, w/v) was diluted in similarly extracted sap of virus-free peppers. The purified CMV (initial concentration 0.68 mg/ml) was diluted in 0.005 M borate buffer pH 9.0 plus 1% EDTA.

TABLE 1. Comparison of absorbance ($A_{405\ nm}$) obtained by using enzyme-linked immunosorbent assay (ELISA) and visual evaluation of symptom severity in cucumber mosaic virus-infected peppers, 3 and 6 wk after inoculation[a]

Cucumber line or cultivar	Symptoms and ELISA absorbance at: 3 wk		6 wk	
	Visual evaluation	$A_{405\ nm}$	Visual evaluation	$A_{405\ nm}$
43	intermediate	0.100	intermediate	0.085
18	intermediate	0.160	intermediate	0.180
66	susceptible	0.230	susceptible	0.160
68	susceptible	0.280	susceptible	0.300
39	susceptible	0.300	susceptible	0.250
13	susceptible	0.390	susceptible	0.190
21	susceptible	0.400	susceptible	0.150
31	susceptible	0.410	susceptible	0.350
Zohar	susceptible	0.410	susceptible	0.350
Maor	highly susceptible	0.430	highly susceptible	0.410
Agronomico-9	highly susceptible	0.450	highly susceptible	0.440

[a]The ELISA results are the means of data from at least 10 plants, after correction for the absorbance of healthy controls. The dilutions were: γ-globulin in coating buffer, 1:500; the conjugated antiserum, 1:2,000; and plant extract, 1:200. The titer of the original CMV-antiserum was 1:2,050 as determined by the ring interface precipitin test.

Since color intensity developing in ELISA could be useful for evaluating relative virus titer(s), providing information which *inter alia* may help in quantifying degrees of resistance (6,7). $A_{405\ nm}$ obtained by ELISA with different dilutions of purified CMV and of crude sap from CMV- or PVY-infected peppers (Fig. 4) showed a close correlation with virus titer. Subsequently, different lines or cultivars of peppers were evaluated for resistance to CMV or PVY by both visual evaluations and ELISA (Tables 1,2). A correlation was found between visual evaluation of resistance and the results of ELISA for both viruses.

TABLE 2. Comparison of absorbance ($A_{405\ nm}$) obtained by using enzyme-linked immunosorbent assay (ELISA) and visual evaluation of symptom severity in potato virus Y-infected peppers, 1 and 5 wk after inoculation[a]

Line or cultivar	Symptoms and ELISA absorbance at: 1 wk		5 wk	
	Visual evaluation	$A_{405\ nm}$	Visual evaluation	$A_{405\ nm}$
Maor	no symptoms	1.384	susceptible	0.316
197	no symptoms	0.849	resistant	0.082
198	no symptoms	1.010	resistant	0.106
199	no symptoms	1.131	resistant	0.196
201	no symptoms	1.134	intermediate	0.214

[a]The ELISA results are the mean of at least 10 plants after correction for absorbance of the healthy controls. The dilutions in the tests made 1 wk after inoculation were: γ-globulin in coating buffer, 1:100; the conjugated antiserum, 1:200; and plant extracts, 1:100. The dilutions at 5 wk were: γ-globulin in coating buffer, 1:300; the conjugated antiserum, 1:500; and plant extracts, 1:100. The titer of the original PVY-antiserum was 1:196 as determined by the precipitin test.

DISCUSSION

The damage caused by viruses in peppers varies inversely with the age of the plant when infection occurs (12). Therefore, where transplanting of seedlings is practiced it is possible to delay and reduce the incidence of infection in the crop by ensuring the use of healthy seedlings. However, screening pepper nurseries for virus incidence has been impractical because of the time required to obtain the results and the difficulty in the mechanical transmission of viruses from pepper (3). The latter also impedes the evaluation of virus titer which could be useful in breeding for virus-resistance (7,16). The results reported in the present work indicate that ELISA may overcome those difficulties. ELISA facilitates the rapid (within 2 days) and accurate detection of viruses in pepper. The sensitivity of the method made it possible to detect virus soon after infection (Fig. 1,2) and to test batches of 50 samples in order to detect a single CMV- or PVY-infected plant (Fig. 3). This makes it possible to assay 1,500 samples in one microplate enabling a rapid and exhaustive screening of viruses in pepper nurseries before planting. Relative CMV or PVY titer could be established by ELISA (Fig. 3,4), and these results could be correlated with resistance levels as evaluated visually (Table 1,2). Moreover ELISA can provide an objective and more detailed evaluation of resistance enabling the discrimination of degrees of resistance, which cannot be achieved visually (Table 1,2).

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