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Detection of beet yellows virus in sugarbeet plants by enzyme-linked immunosorbent assay (ELISA)

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Abstract

Beet yellows virus can be detected in leaf extracts of infected sugarbeet plants by ELISA. The use of discs was studied and proved to be a valuable and qualitatively reliable method. Leaf material could be stored at 4° or 22 °C for at least six days without affecting the detection of this virus by ELISA. A dramatic decrease in ELISA values was found when leaf extracts were frozen.

In an analysis of the distribution of virus over the plant it was found that young leaves present at the moment of infection and those which had still to develop after infection will contain virus. Symptoms produced by systemic virus invasion occur on the oldest leaves containing virus.

Additional keywords: disc method.

Introduction

Beet yellows is a major disease of sugarbeet in all areas where this crop is grown (Duffus, 1977). The disease is caused by two viruses. One, beet mild yellowing virus, is transmitted in the persistent way by the aphid *Myzus persicae* (Russell, 1962), and the other, beet yellows virus (BYV) is predominantly transmitted by *M. persicae* in a semipersistent way (Sylvester, 1961).

The spread of these viruses in the field during the growing season has been the subject of many studies (Jepson and Green, 1983). However, these studies have been hampered by lack of rapid and sensitive methods of testing the presence of virus in plants. As a result, little is known about (1) the distribution of virus in the infected plant, (2) the time after infection at which virus is available for transmission, and (3) to which extent each of the two viruses is spread in the growing season.

We present a study on the application of the enzyme-linked immunosorbent assay (ELISA) for BYV in beet leaf extracts. As the preparation of leaf extracts is a time-consuming process we studied the use of intact leaf discs (Marco and Cohen, 1979; Romaine et al., 1981) instead of leaf extracts in the ELISA (Clark and Adams, 1977). We describe some factors that affect the use of discs and report on the distribution of virus in the diseased plant after infection.

Material and methods

Virus and antiserum. An isolate of BYV kindly supplied by Drs W. Heybroek

(Bergen op Zoom) was maintained in beet plants. For purification young *Claytonia perfoliata* plants were infected by aphids and used two weeks after infection. The virus was partially purified using the method described by Kassanis et al. (1977) and subjected to a further purification by centrifugation on a linear 10-50% sucrose gradient for 3 h at 26 000 rpm in a Beckman SW 27 rotor. The virus zone was collected, diluted with buffer and the virus concentrated by high speed centrifugation into a pellet, which was resuspended in buffer.

Rabbits were immunized with BYV by three injections at intervals of 20 days. The first injection was intravenous with 0.5 mg virus, the others were intramuscular with an emulsion of 1 mg virus in 1 ml buffer and 1 ml of Freund's incomplete adjuvant.

ELISA. The methods used for preparing γ-globulins, conjugating γ-globulins with alkaline phosphatase (Sigma, type VII) and performing ELISA, were described by Clark and Adams (1977). Fractionation of the γ-globulin fraction on a cellulose column after $(NH_4)_2SO_4$ precipitation was omitted.

Polystyrene microtiter plates (M 129 A; Dynatech Laboratories) were coated with 2 μg γ-globulin/ml at 4 °C overnight, unless otherwise stated. Leaf extracts to be tested were prepared from leaf discs with a diameter of 5 mm removed from the leaves with a leather hole punch. The discs were triturated with 0.3 ml phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-Tween) in a small all-glass Elvehjem-Potter tube.

Two modifications of the ELISA technique were also used. In one, the disc method, the use of leaf extracts was substituted by the immersion of one or more leaf discs in the wells of the microtiter plate containing 0.2 ml PBS-Tween. In the other method mixtures of leaf extract and conjugate, or leaf discs immersed in conjugate were incubated at 4 °C overnight (Flegg and Clark, 1979). The γ-globulin concentration in the conjugate was 1.3 μg ml^{-1}. The washings were done with tap water. After incubation with substrate absorbances were measured at 405 nm using a Titertek Multiskan photometer. Duplicate wells were used for each sample and the mean of their A_{405} values was calculated after substracting the absorbance of wells in which no leaf material or conjugate was incubated.

Results

Detection of BYV in purified preparations. The potential of ELISA to detect BYV was determined with purified preparations. Virus concentrations as low as 4 ng ml^{-1} could be discerned (Fig. 1). We confirmed with these results those obtained by Chevalier and Putz (1982). Sap from healthy plants added to purified samples did not interfere with the ELISA readings.

Detection of BYV in leaf extracts of beet plants. Positive ELISA readings were obtained with extracts from infected plants. These extracts prepared by grinding a disc of 5 mm in diameter with 0.3 ml of PBS-Tween could be diluted 256 times to give positive responses. We also investigated the modified procedure described by Flegg and Clark (1979), in which mixtures of leaf extracts and conjugate were incubated. The responses were lower than in the method in which leaf extracts and conjugate were incubated successively in this order. In this modified procedure a prozone effect occur-

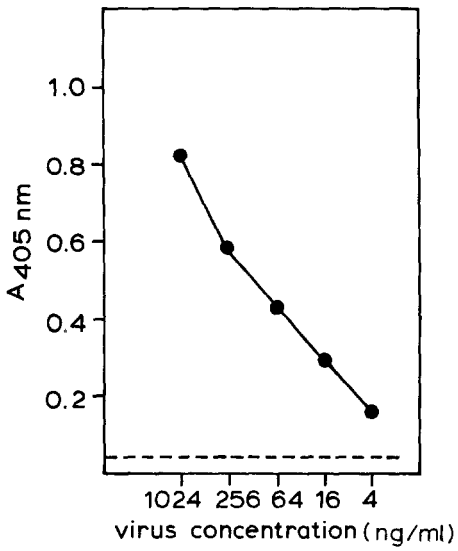

Fig. 1. ELISA absorbance values at 405 nm for purified beet yellows virus diluted with PBS-Tween. A solution of 2 μg γ-globulin/ml was used for coating and the concentration of the γ-globulin in the conjugate was 2 μg ml^{-1}. Background absorbance (---------).

red when a series of sap dilutions were tested. An increase of absorbances was found in the first dilution steps and a decrease if the samples were diluted further.

The use of leaf discs in the ELISA technique. The disc method, which may expedite the preparation of test samples, was used in studies by Marco and Cohen (1979) and Romaine et al. (1981) in detection of potato virus Y and cucumber mosaic virus, and of tobacco ringspot and maize dwarf mosaic virus, respectively.

Fig. 2 shows a comparison of ELISA with discs and with extracts from healthy and infected beet plants, inoculated at different times before testing. The readings obtained with a single disc were lower than those with extracts. The readings varied from 0.08

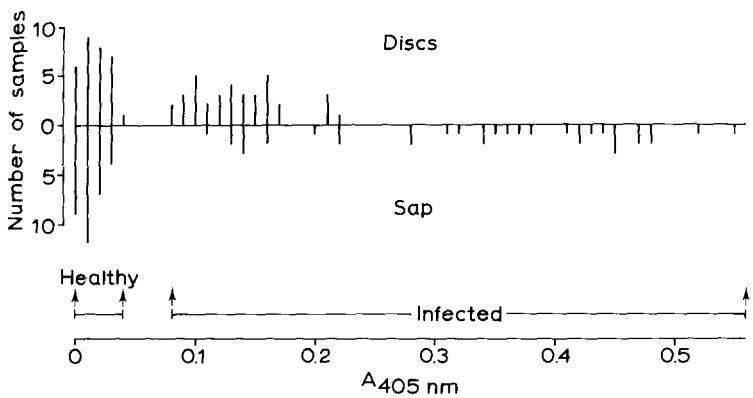

Fig. 2. Comparison of the ELISA using leaf extracts or intact leaf discs as test samples obtained from a single leaf of 36 healthy and infected plants. The plants and infections varied in age. The plate was coated with 2 μg γ-globulin/ml. The samples were incubated for 18 h at 4 °C and conjugate for 4 h at 37 °C.

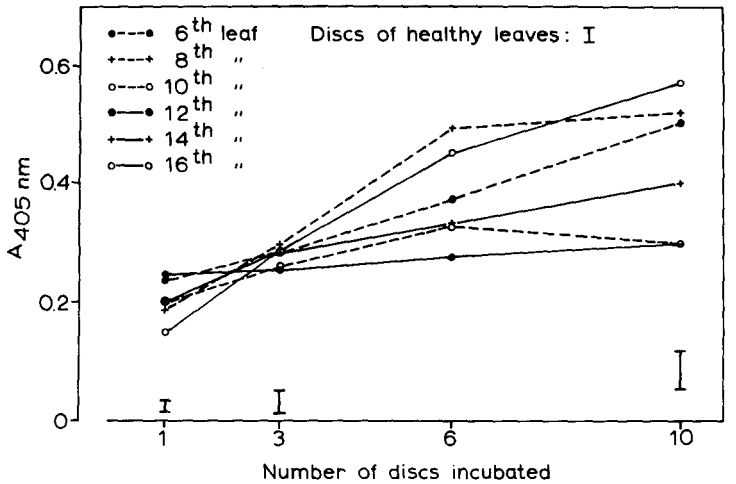

Fig. 3. Relationship between ELISA absorbances (A_{405}) and the number of discs from BYV-infected leaves in the plate wells. The plate was coated with 2 μg γ-globulin/ml. The samples were incubated for 4 h at 37 °C and the conjugate for 18 h at 4 °C.

to 0.220 for discs and from 0.110 to 0.550 for extracts whereas the mean values were 0.136 and 0.329, respectively. No significant differences were found in the readings whether discs or extracts from healthy plants were tested. When an absorbance value twice the A_{405} nm value for healthy sap was taken as a criterium for detection of BYV, a single disc from an infected plant gave a statistically significant positive reaction.

However, for quantitative assessments the method is not suitable because no direct correlation was found between the number of discs immersed and the absorbances measured. This was demonstrated in an experiment in which different numbers of discs from six leaves from one infected plant were tested. Fig. 3 shows that an increase in the number of discs was positively correlated with an increase in absorbance. However, the increase was not proportional to the number of discs used. The results also show that an increase in number of discs used does not significantly improve the detection of BYV.

Several attempts were made to improve the disc method. It is obvious from Fig. 4 that a better discrimination between healthy and infected plants can be reached by the use of higher γ-globulin concentrations in the coating of the plates. An optimum was found for 6 μg/ml. No improvement was found when the discs were vacuum-infiltrated with PBS-Tween or when other buffers were used as antigen buffer. Addition of 0.25% ovalbumin to the conjugate buffer reduced the non-specific reaction found when discs from healthy plants were used. However, ovalbumin was not used in the experiments described in this report.

The use of different incubation schemes can also affect the differences between the readings found for healthy and infected samples. Fig. 5a shows the results of experiments in which the samples and the conjugate were incubated at two different conditions.

Incubation of the extracts and discs at 37 °C for 4 h and the conjugate at 4 °C for 18 h resulted for the infected samples in readings which were about twice as high than

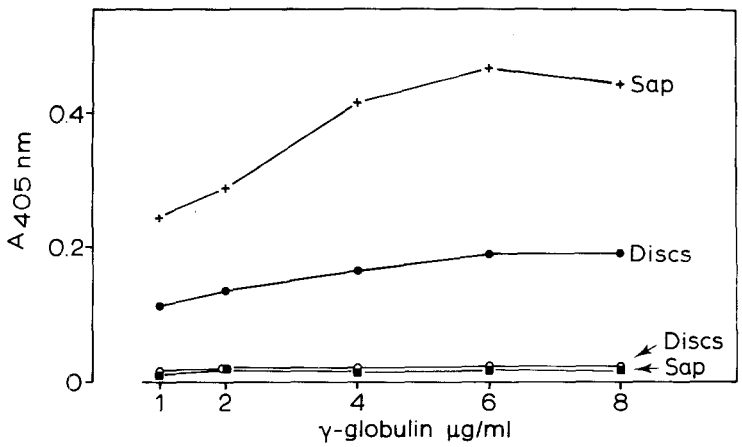

Fig. 4. The effect of different concentrations of γ-globulin, used in the coating, on the detection of BYV in leaf extracts (sap) or intact leaf discs (discs). The samples were incubated for 18 h at 4 °C and the conjugate for 4 h at 37 °C. Two discs were incubated per well; in each experiment two or three wells were used. The A_{405} values given are the mean of the values obtained in three experiments. Sap from healthy (□) and infected (+) plants, discs from healthy (○) and infected (●) plants.

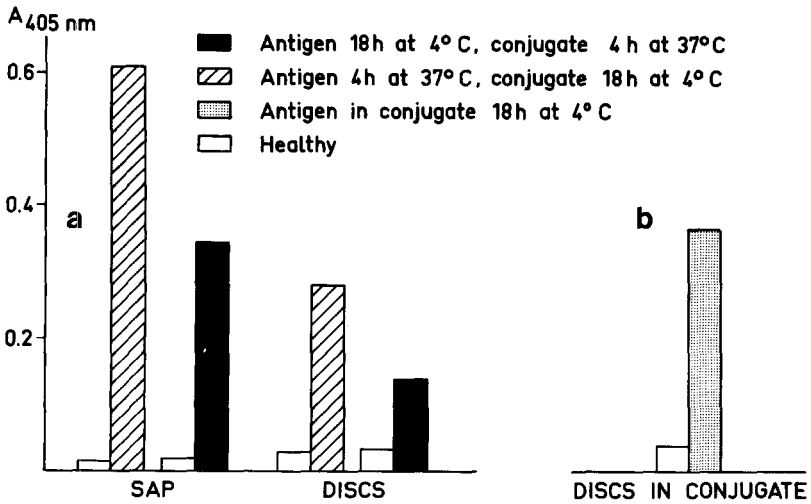

Fig. 5. a) The effect of different incubation conditions for the test samples and conjugate on the detection of BYV in leaf extracts (sap) or intact leaf discs (discs). b) ELISA values obtained after incubation of leaf discs in conjugate solution for 18 h at 4 °C. The plates were coated with 4 µg γ-globulin/ml and two discs were incubated per well. The A_{405} values are the mean of three experiments.

when the incubations were carried out at 4 °C for 18 h and 37 °C for 4 h. In both conditions a large difference was found between the absorbances from infected and healthy material. These results demonstrate again that the disc method is a useful method to detect BYV in beet plants. Even higher readings were obtained when discs and conjugate were incubated together (Fig. 5b). This procedure eliminates two steps viz. macerating the discs and the separate incubation of conjugate in the ELISA procedure.

The effect of storage of infected leaf material on ELISA. Table 1 shows the ELISA values obtained with extracts, discs and leaves, which were stored under different conditions. Storage of leaves placed either with the stems in water or in plastic bags at 4 or 22 °C, did not affect the ELISA. Storage for 6 days usually resulted in higher values. The disc method cannot be used for discs stored in plastic bags. The discs will, presumably, loose so much water during storage that no virus will ooze out into the antigen buffer after immersion. When stored discs were used to prepare extracts, the values were comparable to those which were obtained with stored leaves. The ELISA readings were not affected by storage of sap at 4 or 22 °C. Storage of leaf material at −20 °C reduces these readings slightly whereas dramatic reductions were found

Table 1. The effect of storage of leaf material as leaves, discs and sap at 22°, 4° and −20 °C for 2 and 6 days on the detection of BYV with ELISA. The plates were coated with 4 μg γ-globulin/ml. The samples were incubated at 37 °C for 4 h and the conjugate at 4°C for 18 h.

Treatment during storage	Length of storage (days)	Mean A_{405} values					
		22 °C		4 °C		−20 °C	
		sap[1]	discs[2]	sap	discs	sap	discs
Leaves placed with the stem in water	0	0.72	0.62	0.66	0.78	—	—
	2	0.75	1.07	0.70	0.89	—	—
	6	1.32	1.60	0.92	1.06	—	—
Leaves stored in a plastic bag	0	0.76	0.71	0.80	0.57	0.74	1.00
	2	0.76	0.73	0.64	0.58	0.51	0.96
	6	1.89	2.00	0.95	0.98	0.48	0.63
Discs stored in a plastic bag	0	0.74	0.77	0.74	0.77	0.74	0.77
	2	0.82	0.22	0.70	0.21	0.45	0.55
	6	1.20	0.26	0.77	0.25	0.37	0.35
Extract[3]	0	0.74	—[4]	0.74	—	0.74	—
	2	0.64	—	0.75	—	0.39	—
	6	0.63	—	0.57	—	0.18	—

[1] Sap: an extract of a disc was used as antigen sample.
[2] Disc: a disc was used as antigen sample.
[3] Prepared as described in Material and methods.
[4] Not tested because the experimental layout did not provide for these samples.

when sap was stored at −20 °C. The ELISA values of samples from healthy material did not change during storage.

Finally, it was noted during this experiment that removal of discs was easier from leaf material stored at 4 °C or on water than from leaf material stored otherwise.

Distribution of virus in sugarbeet plants. To demonstrate which leaves have to be used for the detection of BYV we investigated the distribution of virus in the plant in connection with the development of disease symptoms on the plant. A summary of this study has already been published (Roseboom and Peters, 1983). A series of plants each of which had approximately 25 leaves were infected by allowing viruliferous aphids to feed on leaves which were either old, middle-aged or young. Subsequently, the foliage of these plants was analyzed periodically. High levels of virus detected with

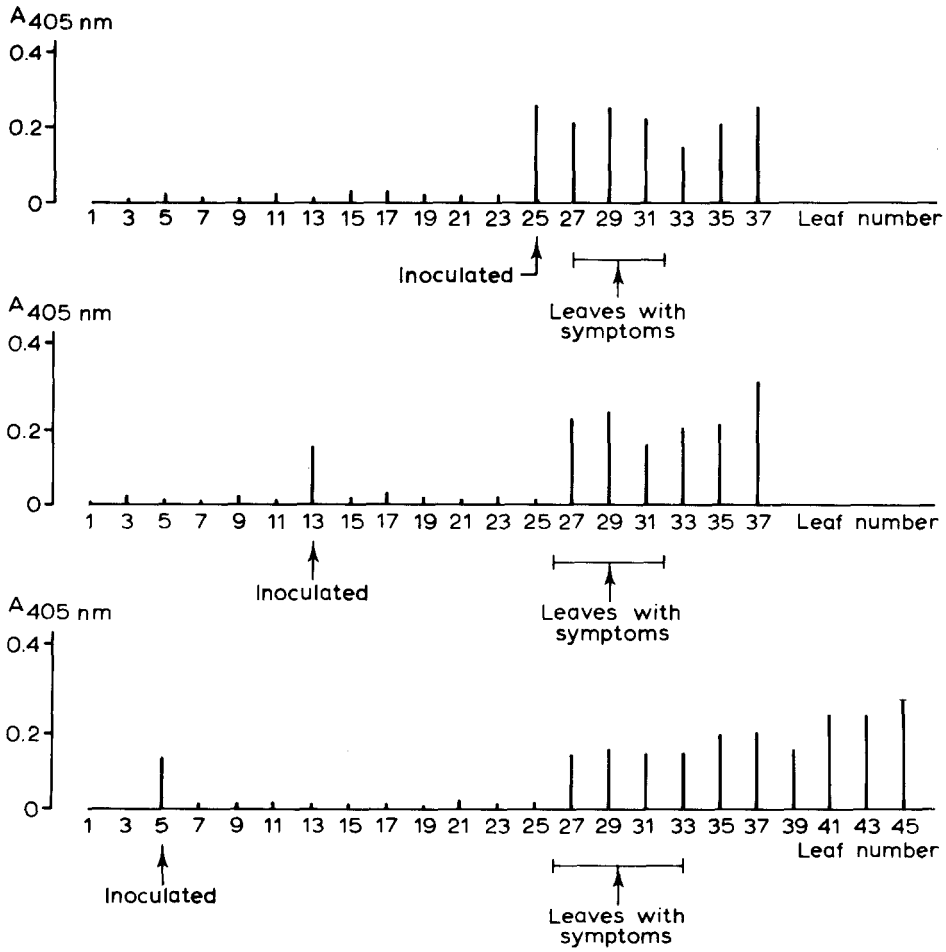

Fig. 6. The distribution of virus and symptoms over the foliage of sugarbeet plants after infection of leaves of different ages with BYV. The plants inoculated at the old leaves were sampled one and a half months after inoculation, the plants of the other groups after one month.

ELISA were (1) in the inoculated leaves, (2) in the leaves which at the time of inoculation had grown to only approximately half of their final length, or less, and (3) in all leaves developing after inoculation (Fig. 6). Interestingly, very little or no virus was detected in those leaves that were fully grown at the time of inoculation, nor did those leaves develop disease symptoms. Thus it appears that the virus is not transported through or occurs in the whole plant, but that it only replicates in growing and developing leaves and in the leaf inoculated. This distribution of virus over the plant as determined with ELISA was confirmed by infectivity tests with aphids. The results of this study demonstrate that when ELISA is used to detect BYV infection, plant samples should be taken from young leaves because they most likely will contain the highest levels of virus. The yellowing symptoms characteristic of the disease developed on those leaves which had nearly reached half their length or less at the time of infection and in which high levels of virus were eventually detected, as well as on the leaves used to inoculate the plant.

Discussion

ELISA provides a reliable method to detect BYV in infected sugarbeet plants. However, results obtained in our studies on the distribution of virus in the plant show that the youngest leaves should be sampled. In preliminary experiments we determined that virus could be detected with ELISA 7 to 9 days after inoculation, which is one to three weeks before symptoms develop.

The results obtained with discs from infected leaves show that the disc method can detect BYV at a level of sensitivity which is useful for practical and qualitative tests and for indexing large plant populations. The sensitivity can be improved by increasing the concentration of γ-globulin in the coating step, application of ovalbumin and by changing the incubation conditions. Other improvements may be obtained by optimizing enzyme labeling, and by reducing the non-specific colouring of samples from healthy plants. Additionally, it may be possible to improve virus detection by modifying the method by which the discs are cut from the leaves.

Discs from yellow areas of leaves with symptoms often gave higher readings than those from green areas. The differences in ELISA values were minor when discs were tested from different areas of infected young leaves without symptoms. However, the values may differ considerably for the various young leaves of one plant, and also for those of different plants. As a disc or an extract from a young infected leaf may occasionally give a low reading, it is advisable to test two or three discs from a leaf in a well.

A single disc can be used to detect an infected plant. Increasing the number of discs per well may lead to an absolute increase in the differences between the readings of samples from healthy and infected leaves, but no proportional relationship was found between the ELISA absorbances and the number of discs immersed per well, as has also been found by Marco and Cohen (1979) and by Romaine et al. (1981).

The disc method cannot be used quantitatively. To determine the virus concentration, leaf extracts should be used. The disc method will not likely prove useful for measuring the virus present in leaf material even when cutting of discs will be improved.

Samenvatting

Detectie van het bietevergelingsvirus in bieteplanten met ELISA

Het bietevergelingsvirus kan op betrouwbare wijze met de ELISA methode in geïnfecteerde bieteplanten worden aangetoond. Een aanzienlijke vereenvoudiging van de procedure kan worden bereikt met de zogenaamde 'disc-method', waarbij intacte ponsstukjes in de putjes van de ELISA-plaat worden geïncubeerd. Hierbij komt voldoende virus uit de ponsstukjes voor ELISA vrij. Bladmateriaal kon op verschillende wijzen bewaard worden zonder dat de mogelijkheid om het virus aan te tonen achteruitging. Met bladextracten die ingevroren waren, werden echter slechte resultaten verkregen.

In een analyse naar de verdeling van het virus over het loof bleek het virus voor te komen in de geïnoculeerde bladeren, in die bladeren die op het tijdstip van inoculatie minder dan de helft van hun uiteindelijke lengte bereikt hadden en in de bladeren die nog moesten verschijnen. De symptomen ontwikkelden zich op de oudste systemisch geïnfecteerde bladeren.

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