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The detection of three viruses of hop (*Humulus lupulus*) by enzyme-linked immunosorbent assay (ELISA)

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SUMMARY

The recently described technique of enzyme-linked immunosorbent assay (ELISA) was used throughout the 1976 growing season to detect hop mosaic, arabis mosaic and prunus necrotic ringspot viruses in hop plants. On each occasion virus was detected quickly, conveniently and with great sensitivity. The technique was particularly suitable for processing numerous samples collected from the field.

Serious difficulties and limitations were encountered in testing comparable material by established techniques. The serology test for the hop strain of arabis mosaic virus by double diffusion in agar gels was very insensitive and only worked satisfactorily early in the growing season. Grafting sensitive Golding hop varieties to detect hop mosaic virus was inconvenient and time-consuming and symptom expression was so slow and erratic that glasshouse space was utilized for long periods. It became impossible to detect prunus necrotic ringspot virus by sap inoculations to cucumber during an exceptionally hot period in mid-summer.

The possibilities are discussed of exploiting the ELISA technique for use in large scale surveys and epidemiological studies on viruses of hop and other crops. Changes in the current methods of handling and extracting leaves are considered for increasing the throughput of samples.

INTRODUCTION

Arabis mosaic virus (AMV), prunus necrotic ringspot virus (NRSV) and hop mosaic virus (HMV) are widespread and prevalent in the hop (*Humulus lupulus* L.) in England and some other countries. However, the incidence and spread of these viruses is difficult to assess because they seldom cause obvious symptoms. In England NRSV symptoms are rare, HMV is always latent except in sensitive Golding varieties, and only some of the plants containing AMV develop nettlehead and/or severe split leaf blotch.

Serology and inoculation of suitable indicator plants have both been widely used to detect latent infection in hop, but existing techniques are insensitive or have other serious defects that restrict their use. These constraints have been overcome by using the recently described enzyme-linked immunosorbent assay (ELISA) technique for detecting plant viruses (Voller *et al.* 1976; Clark & Adams, 1977). This paper describes the use of ELISA in detecting latent virus infection of hop and considers the suitability of the method for large scale use in surveys and epidemiological studies.

MATERIALS AND METHODS

The ELISA tests were done in polystyrene microtitre plates using the methods and antisera of Clark & Adams (1977). All plant extracts were prepared by grinding weighed quantities of tissue (usually 0·2 g) in a pestle and mortar with phosphate buffered saline containing 0·5 ml/l Tween 20 and 20 g/l polyvinylpyrrolidone (M.W. 24500 or 44000). Except where stated, extracts were prepared at a standard dilution of 1:50 (w/v) and all plates were loaded within 5 h.

Positive ELISA reactions produce a yellow coloration due to the action of antibody-linked alkaline phosphatase on the *p*-nitrophenyl phosphate substrate; negative reactions remain colourless. The results can be expressed quantitatively by measuring absorbance at 405 nm.

For comparisons with ELISA, AMV was usually assessed serologically by double diffusion tests in agar gels using undiluted sap (Bock, 1966). When testing for AMV and NRSV by sap inoculation, extracts were prepared by grinding young hop leaves and shoots in a pestle and mortar with 0·03 M phosphate buffer (pH 8·0) usually containing 10 g/l polyethylene glycol (M.W. 6000). Extracts were prepared at a standard dilution of 1:4 (w/v) and were immediately inoculated manually, using Carborundum as an abrasive. Cucumbers cv. Ohio were inoculated at the cotyledon stage, using three plants in one pot for each sample to be tested for NRSV. For AMV at least two plants of *Chenopodium amaranticolor* Coste & Reyn. and/or *Chenopodium quinoa* Willd. were inoculated at the eight-leaf stage. The herbaceous test plants were usually raised and maintained in a heavily shaded glasshouse subject to considerable temperature fluctuations. In some tests the plants were kept after inoculation in an air conditioned chamber at 18 °C.

Various procedures were used to detect HMV in hop by grafts to sensitive hop cultivars. The usual method involved bottle or chip grafts to mist propagated plants cv. Bramling, but some tests were done by chip grafts to single node cuttings which were then rooted in flasks of water or under mist (Schmidt, 1965).

RESULTS

Arabis mosaic virus

AMV is more widespread in the hop in England than in any other known host, although its occurrence and prevalence were for long overlooked (Thresh, Pitcher, McNamara & Ormerod, 1972). This is because infected hop plants do not always develop obvious symptoms and the strain of AMV usually occurring in hop has very slight effects on herbaceous test plants compared with isolates from other hosts. Consequently AMV in hop has usually been detected serologically by double diffusion tests in agar.

The limitations and insensitivity of this procedure were emphasized in recent investigations done for comparison with ELISA. Initially, plants were sampled individually in March 1976 by collecting emerging buds at a site in Kent where AMV is rife. Standard extracts either did not react (mean $OD_{405} = 0{\cdot}060 \pm 0{\cdot}004$) or reacted strongly (mean $OD_{405} > 2.000$) in ELISA tests and it was then established that the

technique was discriminating between AMV-infected and uninfected plants. Considerable difficulty was encountered in obtaining the necessary confirmatory evidence by double diffusion serology or by inoculating *Chenopodium* spp. The serological reactions in agar were faint, developing consistently only in tests done on undiluted extracts of buds and occasionally at dilutions of 1:2 and 1:4. Moreover, the inoculated *C. amaranticolor* plants remained symptomless and the symptoms in *C. quinoa* were so inconspicuous and transient that all infections had to be confirmed serologically by double diffusion tests on sap obtained 4 wk after inoculation.

Developing buds collected in spring were convenient for testing by ELISA as they were small and compact and yet contained several leaves and leaf primordia. Extracts were prepared more readily than from mature leaves collected later and the ELISA values obtained with infected samples were consistently high.

Table 1. *Mean fresh weights and ELISA values for tests on the different parts of ten developing buds collected from arabis mosaic virus-infected hop plants on 24 March 1976*

	Fresh weight (g)		ELISA values (OD_{405})	
Tissues	Mean	S.E.	Mean	S.E.
Growing point, etc.	0·052	0·087	0·406	0·117
Leaves	0·022	0·002	0·440	0·127
Bud scales	0·115	0·016	0·239	0·069
Stem	0·817	0·062	0·129	0·037

The use of ELISA for assessing the virus content of small quantities of tissue was demonstrated in tests on samples of infected buds collected from the field and dissected into their constituent parts. The young leaves and growing point comprised only a small proportion of the total fresh weight but they contained much higher concentrations of detectable virus than the scale leaves and stem (Table 1).

The double diffusion test is so insensitive that when assessing spread of AMV in field trials each plant must be tested individually. By contrast bulk sampling was possible by using ELISA for the 1976 assessments, with great savings of time and effort. In a trial at Rosemaund Experimental Husbandry Farm, Hereford, the experimental plants were sampled individually only if AMV was detected in the initial tests done on bulked samples from the ten plants of each subplot. Using this procedure the 980 plants in the trial were assessed by testing only 178 samples.

Past experience has shown that the double diffusion serology test becomes increasingly unreliable as the season progresses, especially during and immediately after prolonged periods of hot sunny weather. This trend was particularly obvious during the exceptionally hot summer of 1976. It then became virtually impossible to detect AMV, even in plants with conspicuous symptoms of nettlehead disease that earlier had yielded saps reacting at dilutions of up to 1:4 or 1:8 in agar gels. By contrast, ELISA was used successfully throughout the growing season and strong specific reactions were obtained with apparently intractable material such as old discoloured leaves bearing extensive spray deposits. Stronger reactions were obtained in July with such leaves than with young leaves and shoots (Table 2).

Table 2. *Representative ELISA values for assays on leaves collected from healthy and arabis mosaic virus (AMV)-infected hop plants on* 20 *July* 1976

Leaves*	ELISA values (OD_{405}) AMV-infected	Healthy
Old (N)	0·894	0·007
Old (O)	0·582	0·006
Intermediate (O)	0·218	0·029
Young (O)	0·198	0·024
Tip (O)	0·056	0·015

* The leaves from the AMV-infected plants were with (N) or without (O) conspicuous symptoms of nettlehead disease. All extracts were prepared at standard 1:50 dilutions.

Hop mosaic virus

Detecting HMV in hop by grafts to sensitive indicators requires considerable skill, and glasshouse space is utilized for weeks or sometimes months to ensure that all infected plants have developed symptoms. These difficulties were emphasized in 1975 in monitoring the spread of HMV into the plots of a field trial at Wye College. Grafting samples from the 180 experimental plants commenced on 29 May and continued sporadically for 3 months. On very hot days it was difficult to transport the samples the 30 miles back to East Malling in satisfactory condition and it was impracticable to handle more than about thirty grafts per occasion. Some inoculated plants died or the grafts failed and had to be repeated. Suitable indicator plants or glasshouse space were not always available when required.

Symptom expression was erratic and inconsistent. Some plants developed conspicuous symptoms within 4 wk of grafting. Others did not develop symptoms for several months or in some instances until after they had been dormant throughout the winter of 1975–6. By March 1976, grafts from 113 of the 180 experimental plants had caused symptoms in the Golding indicators. Some of the inoculated plants with symptoms and all symptomless plants were then tested by ELISA. Extracts from uninfected control plants did not react (mean $OD_{405} = 0{\cdot}018 \pm 0{\cdot}003$), whereas there were strong positive reactions (mean $OD_{405} = 1{\cdot}607 \pm 0{\cdot}068$) with extracts from all plants with mosaic symptoms and from seven of the symptomless plants. These plants developed symptoms during the ensuing weeks, whereas plants negative in the ELISA test did not.

The ELISA technique was equally successful in detecting HMV in mosaic-tolerant varieties that produce no visible symptoms of infection. Invariably there were strong reactions (mean $OD_{405} = 1{\cdot}126 \pm 0{\cdot}146$) with extracts from infected plants, but not with extracts from virus-free plants derived from meristems (mean $OD_{405} = 0{\cdot}015 \pm 0{\cdot}004$). ELISA was used later to assess the spread of HMV into a large trial of meristem clones of mosaic-tolerant varieties at Rosemaund E.H.F. Two people processed 264 samples within 2 days in testing each plant individually and also after grouping into batches of ten to assess the possibilities of bulk sampling procedures. The individual samples either did not react or gave strong positive readings. The results for the bulk samples gave a generally good agreement between the numbers of infected plants

as estimated from the absorbance values (y) and the number known to be infected from the individual tests (x). The curvilinear relationship between x and y can be expressed mathematically by an equation which accounts for 94% of the variance:

$$y = 2{\cdot}53\ (1 - e^{-0{\cdot}43x}),$$

where the constant $e = 2{\cdot}7183$ (the base of Napierian logarithms).

The 288 plants of various seedling progenies in plots at Wye College were tested several times in 1976. At the first assessment in June the overall levels of infection in progenies planted out in May 1974, 1975 or 1976 were 52, 22 and 2 %, respectively. In subsequent tests the original infections were confirmed, but there were few additional ones, even in the test done at the end of the growing season on 7 October. This suggests either that there is little spread of HMV after mid-summer or that infection is slow to become systemic and increase to a detectable level. These possibilities will be investigated further by ELISA in routine tests throughout the season to follow the pattern and sequence of spread.

Table 3. *ELISA values obtained in tests on dilutions of extracts made by a partial or thorough grinding of leaf samples collected from hop mosaic virus (HMV)-infected and healthy hop plants*

	HMV-infected		Healthy	
Extract tested at*	Partially ground	Thoroughly ground	Partially ground	Thoroughly ground
1:10	1·499	2·000	0·037	0·100
1:100	0·238	0·856	0·066	0·066
1:320	0·170	0·355	0 085	0·060
1:1000	0·053	0·239	0·035	0·030

* The dilutions and assays were done after pooling standard (1:50) extracts made from ten different samples collected on 27 Oct. 1976.

In 1976 much time was spent in weighing and grinding the samples and for at least some purposes throughput might be increased by a simplified procedure. Encouraging results were obtained in tests done on old leaves collected from outside plots in mid-October. Extracts were then prepared by grinding the leaf samples thoroughly in buffer until only very small fragments of tissue remained intact or for $\frac{1}{2}$, $\frac{1}{4}$ or $\frac{1}{8}$ the mean time of 40 s required for such complete disintegration. Specific reactions were obvious (mean $OD_{405} > 0{\cdot}500$) with all samples and the extracts ground least were the only ones that did not give very high readings.

In further tests samples were ground partially or more thoroughly using from half to four times the standard amount of 0·2 g of leaf per 10 ml of buffer. The degree of grinding and leaf/buffer ratio were not critical and intense reactions were obtained with all extracts. Those prepared by thorough grinding contained more virus than extracts prepared in only half the overall time using the quick procedure (Table 3). However, the difference was not apparent visually and the reactions were too intense to be measured photometrically until the extracts were diluted further. No infections would have been missed by a visual assessment of extracts prepared by incompletely grinding samples, even if these were not of the exact weight. If there is similar latitude

in tests done earlier in the growing season it will be unnecessary to weigh the samples or to grind them thoroughly in routine tests for the presence or absence of virus. This will save time and effort and permit an increased throughput of samples.

Prunus necrotic ringspot virus (NRSV)

Young cucumber plants inoculated mechanically with NRSV from hop usually develop conspicuous symptoms within 3–8 days. The procedure is quick and convenient and has been used to follow the spread of NRSV in hop (Thresh & Ormerod, 1974). However, the cucumber test does not entirely obviate the need for serology,

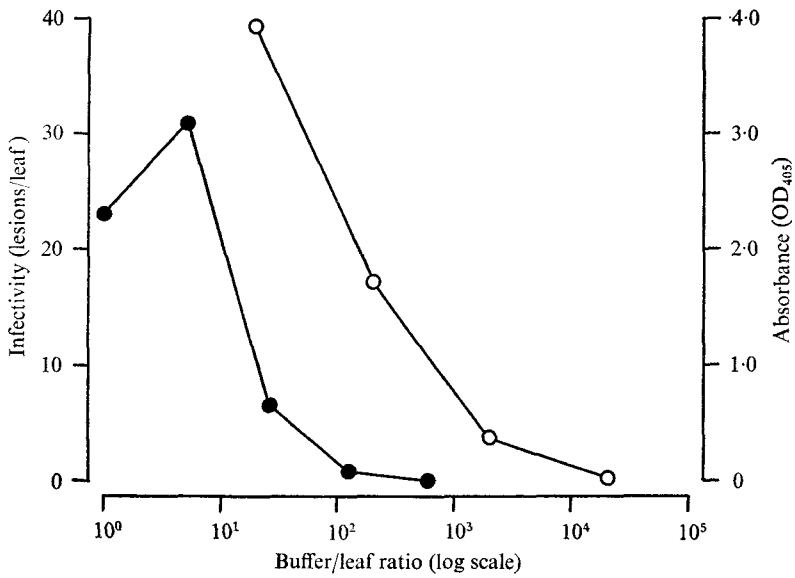

Fig. 1. The detection of prunus necrotic ringspot virus in hop by inoculation to cucumber (●) and by ELISA (○) using extracts made at different buffer/leaf ratios.

because the symptoms of NRSV in cucumber are not diagnostic and may resemble those of other viruses. These include the type strain of AMV (Murant, 1970) and cucumber mosaic virus, which has been isolated from hop in England (Bock, 1966) and East Germany (Schmidt & Karl, 1968). Another disadvantage of the inoculation procedure is that the susceptibility of cucumber test plants decreases rapidly with age. Much glasshouse space and labour are required to produce the continuous succession of young well-grown plants needed for routine tests and inevitably some plants are wasted.

Work with ELISA began in the spring of 1976 when field samples were collected from various plantings at Wye College that had been established several years previously with NRSV-free material. Inoculations to cucumber and ELISA tests then gave similar results. The only discrepancies arose from an initial failure to transmit NRSV from a few plants that gave definite ELISA reactions. In all such instances NRSV was detected by further inoculations.

On 13 May 1976, NRSV was readily detected by inoculating cucumbers with hop leaf extracts prepared at dilutions up to 1:125 (w/v), while the ELISA reactions were clearly visible at 1:2000 and were detected photometrically at 1:20000 (Fig. 1). Three weeks later during a prolonged period of hot sunny weather the ELISA reactions were still strong, whereas cucumber plants were not infected by extracts at 1:4 whether the test plants were grown in the usual way or were kept at 18 °C before and after inoculation. Temperatures were less extreme by the end of July when NRSV was transmitted from the shoot tips of infected plants and from young and intermediate leaves, but not from old leaves or flowers. By contrast, all infected tissues gave specific ELISA reactions, although these were sometimes weak with extracts of very old leaves.

Table 4. *Mean ELISA values obtained in tests for prunus necrotic ringspot virus in thirty-six varieties sampled twice at Wye College in* 1976

		ELISA values (OD_{405})		
Date (1976)		Strong (n = 10)	Weak (n = 16)	None (n = 10)
27 May	Mean	3·42	0·68	0·01
	S.E.	0·20	0·04	0·01
20 July	Mean	> 2·00	0·55	0·02
	S.E.	—	0·04	0·01

A feature of the NRSV results was that the ELISA values obtained with standard extracts from individual plants were more variable than those obtained in tests with other viruses. Some hop plants gave consistently strong reactions, whereas others gave weaker ones (Table 4). Virus concentration effects could have been involved, but similar results were obtained in later retests and in tests done after transferring the strong and weak reacting isolates to cucumber. This suggests that the antiserum used in this work, which was prepared against the Paradise apple mosaic virus serotype of NRSV (Fulton, 1968), was distinguishing between strains. Such differences have been reported in previous studies on isolates from hop using conventional serological techniques (Bock, 1967). This possibility and the use of ELISA to assess serological relationships are now being considered.

DISCUSSION

These experiments have established the value of ELISA for the detection and assay of viruses of hop with a speed and on a scale hitherto impracticable. Tests for HMV, NRSV, and AMV can now be done concurrently on different aliquots of the same extract and throughout the growing season. There are major savings of time and effort and recurring requirements for glasshouse space and test plants are avoided. Additional merits of ELISA are its versatility and sensitivity and its suitability for use with very small samples of tissue.

At East Malling ELISA has now superseded existing methods of detecting hop viruses, so eliminating a major constraint on the scale, timing and sensitivity of

surveys and experiments. The number and size of field experiments on the epidemiology and control of hop virus diseases can now be greatly increased without increasing the assay work involved. Bulk sampling procedures are feasible using appropriate statistical techniques (Gibbs & Gower, 1960) and it is now possible to plan regional surveys on an adequate scale to assess the prevalence of AMV in commercial plantings.

The main limitations so far encountered in testing numerous samples by ELISA concern the logistics of collecting, labelling, transporting, storing and handling the material before assay. Much laboratory time is spent in subsampling and weighing, but for many purposes the leaf: buffer ratio is unlikely to be critical and it may be unnecessary to weigh or label samples individually.

The main bottleneck is in preparing extracts, especially when the plant material is tough and difficult to grind. To date, almost all extracts have been prepared manually using a pestle and mortar. This is tedious and slow, although for some tests it will suffice to grind the samples less thoroughly than has become customary. An electrically driven grinder gave satisfactory results but was noisy and had to be washed carefully between samples. Nevertheless, there is considerable scope and good prospects for partial or complete automation. A suitable apparatus is available for extracting sap from potato leaves for agglutination tests and further exploratory work is justified with this and other possible devices. Assay presents no difficulties and a throughput far greater than anything yet achieved is possible, especially if a visual assessment of the results is adequate or if the absorbance values are determined automatically.

From our initial experiences with viruses of hop and also of woody plants (Clark, Adams, Thresh & Casper, 1977) it is already clear that there are many other possible applications of ELISA for surveys and in field and laboratory studies. The technique is particularly well suited for studying the incidence and spread of viruses causing latent infections, or symptoms that are inconspicuous, transient or otherwise of limited diagnostic value. When suitable antisera are available to several of the viruses likely to be encountered in a particular crop there are obvious advantages in being able to run concurrent tests with the same extracts. ELISA has obvious potential with a crop such as potato to supplement or replace existing procedures for virus-testing foundation stocks and for assessing the suitability of field grown material for use the following season. There is a regular and extensive deployment of technical personnel and glasshouse or other facilities for this work in Britain and many other countries.

In work on specific diseases, ELISA is now being used extensively in England and Germany for the quick and accurate diagnosis of plum pox, which should facilitate control by eradication. Other possible applications are in seed testing and in monitoring the reinfection of the virus-free material of many crops now being supplied for official certification schemes and commercial plantings.

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