

THE DIAGNOSIS OF *PLUM POX VIRUS* IN THE UK: FROM STRAIN DIFFERENTIATION TO ON-SITE DETECTION

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Keywords: RT-PCR, RFLP, lateral flow device, potyvirus, *Potyviridae*

Abstract

Plum pox virus (PPV) is a highly damaging virus of *Prunus* spp. This pathogen was first recorded in the UK in the 1960s and rapidly established itself in plum crops in England. As part of an on-going campaign to control this virus, accurate diagnosis is vital and new methods have been recently introduced to support existing ELISA tests. These include IC-RT-PCR with RFLP analysis, which permits accurate strain differentiation. Using these methods, all ELISA positive samples are now confirmed and strain-typed. To date only the D-strain has been detected. In addition to laboratory testing, an on-site test kit has also been developed for use during field inspections. This kit, which consists of a one-step lateral flow device and a simple, bottle extraction system, can give a result in less than 3 minutes.

1. Introduction

Plum pox or Sharka is a highly damaging disease of *Prunus* spp., which is caused by *Plum pox virus* (PPV; Family *Potyviridae*, Genus *Potyvirus*). Originating in eastern Europe, the disease was first recorded in the UK in 1965, on plum trees (*Prunus domestica*) in Kent, England (Pemberton, 1980). However, despite a vigorous campaign, eradication was not achieved, with the disease reappearing in 1970. Over the following decade, PPV became established in all the main plum growing areas of England and was also found on damson (*Prunus domestica* subsp. *insititia*) (Pemberton, 1980).

With full eradication proving impossible to achieve, alternative measures have been adopted to mitigate the effects of the disease in the UK. Amongst these measures is the regular monitoring of propagators and the production of PPV-free material. This is currently achieved through a combination of visual field inspections and laboratory testing, done by ELISA. However, this system has limitations, in particular with the inability to differentiate between the various PPV strains present in Europe. With the spread of more aggressive PPV-strains (e.g. M-strain) through Europe (Poggi Pollini *et al.*, 1997) and the discovery of the Cherry strain (Nemchinov *et al.*, 1998), the ability to identify PPV to strain has become important. In addition, over recent years there has been a move towards the development of reliable on-site test kits, for use by inspectors in the field. By using this type of technology, the speed and cost of diagnosis can be greatly reduced. This paper describes the work done by the Central Science Laboratory (CSL) in the UK, to establish routine strain typing and to develop on-site test kits for field diagnosis.

2. Materials and methods

2.1. PPV isolates

Type virus isolates (PPV, PVY etc) were maintained on either *Prunus domestica*,

Nicotiana benthamiana or *N. tabacum*, in either an insect-proof screenhouse (*Prunus* spp.) or glasshouse (*Nicotiana* spp.). Other test samples were those received by the virology diagnostic laboratory based at CSL, during the summer seasons from 1998 onwards.

2.2. Double antibody sandwich (DAS) ELISA

Tests were performed using a rabbit anti-PPV polyclonal antiserum, produced at CSL. An alkaline phosphatase conjugate was made using the same antiserum, using a Linkit AP-conjugation kit (ISL Ltd). ELISA was performed using a standard DAS method, adapted from Clark and Adams (1997).

2.3. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

IC and single-tube RT-PCR were performed as described by Mumford and Seal (1997), using 0.2 ml PCR tubes coated with 1:500 (v/v) PPV polyclonal (produced at CSL) and the universal PPV primers described by Wetzel *et al.* (1991).

2.4. Restriction fragment length analysis (RFLP)

RT-PCR product analysis was performed using *Rsa* I and *Alu* I enzymes (Promega), as recommended by Wetzel *et al.* (1991). After RT-PCR, separate digests for each enzyme were set up, by adding 2 μ l (20 U) of enzyme directly to 18 μ l of RT-PCR. The tubes were then incubated at 37°C for 2 hours, before analysing by electrophoresis, using either a 4% (w/v) Metaphor (FMC) or 2% agarose (w/v) TBE gel (the latter was used routinely).

2.5. Lateral flow devices (LFD)

A one-step LFD was developed using a PPV polyclonal (produced at CSL) for the immobilised target line and also for coating the latex particles (Fig. 1). An anti-rabbit antibody (Sigma) was used for the immobilised control line. The finished devices were used in conjunction with sap extracted from either a piece of leaf measuring approximately 1 cm square or using 20 leaf-discs, cut from 10 separate leaves. Extracts were made using a 'bottle and ball' method, where the test sample is placed in a small, plastic, screw-top dropper bottle containing 5 ml of extraction buffer and several small, ball bearings. The bottle is then shaken vigorously for around twenty seconds to extract the sap, before applying 2-3 drops into the sample well of the test device. The result is read after 3 minutes. Interpretation is based on obtaining two lines (T and C) for the presence of pathogen, whilst one line (C only) indicates that the pathogen was not detected, but the device worked correctly.

3. Results and discussion

Since 1998, all PPV ELISA-positive samples detected at CSL, have been confirmed and strain-typed by IC-RT-PCR and RFLP analysis. To date eleven samples have been tested and all of these have been confirmed as the D-strain of PPV (Table 1). With the small number of samples currently being tested, RT-PCR is an ideal confirmatory method, not only because it is fast and sensitive, but it also provides a simple means to distinguish different PPV strains accurately (Fig. 3). This method also has the advantage of being based around a pair of primers that have been extensively tested and which have proved consistently reliable for detecting and differentiating between all the three main strains of PPV, namely D, M and C (Poggi Pollini *et al.*, 1997; Nemchinov *et al.*, 1998).

In addition to providing reliable detection and differentiation, the PCR-RFLP

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method used also includes a number of steps which greatly reduce the amount of staff time required; a significant consideration for a routine diagnostic method. This includes the IC step, which removes the need for more traditional, complicated RNA extractions to be performed. The one-step RT-PCR also eliminates the requirement for separate cDNA synthesis reactions to be performed. Finally, as both of the restriction enzymes used work efficiently in the Taq buffer used for RT-PCR, digests can be performed directly without the need for prior PCR product purification. Again, this significantly streamlines the whole RFLP analysis procedure. Although, as mentioned earlier, only a small number of samples currently have to be tested by RT-PCR, the ease-of-use of the current method would make it readily amenable to scaling-up, allowing considerably more samples to be tested and without the need for extra staff.

In addition to molecular testing, a one-step LFD that allows the rapid and specific detection of PPV in the field, has also been developed. When tested against PPV-infected *Prunus domestica* and *N. benthamiana*, clear positive results were achieved within 3 minutes of sap being applied to the device. In contrast, the LFD gave no test-line reaction for either uninfected leaves or material infected with other potyviruses, including potato viruses A, V, Y (O- and N-strains) and *Turnip mosaic virus* (results not shown). Overall the sensitivity of the LFD is around 4 times less than that of DAS-ELISA, which uses the same polyclonal antibody (results not shown). However despite this, the LFD is capable of detecting a single, symptomatic infected leaf within a total sample of 10 plum leaves (Fig. 3). Being able to attain this level of sensitivity is particularly important, as it is well established that PPV can be unevenly distributed throughout an infected plum tree, and testing bulked samples is often required (e.g. for routine DAS-ELISA testing, bulked samples consisting of twenty four, randomly-sampled leaves per budwood mother tree are used). At present, the LFD kits are being evaluated by the Plant Health and Seeds Inspectorate (PHSI) in the UK. If this trial proves successful, these devices will be routinely issued to inspectors and should significantly improve the precision of detection in the field, which currently relies on visual assessment. The possible role of field test kits in certification schemes for PPV would require further evaluation.

Acknowledgments

This work was carried out in part under a memorandum of understanding between MAFF Plant Health Division and CSL.

References

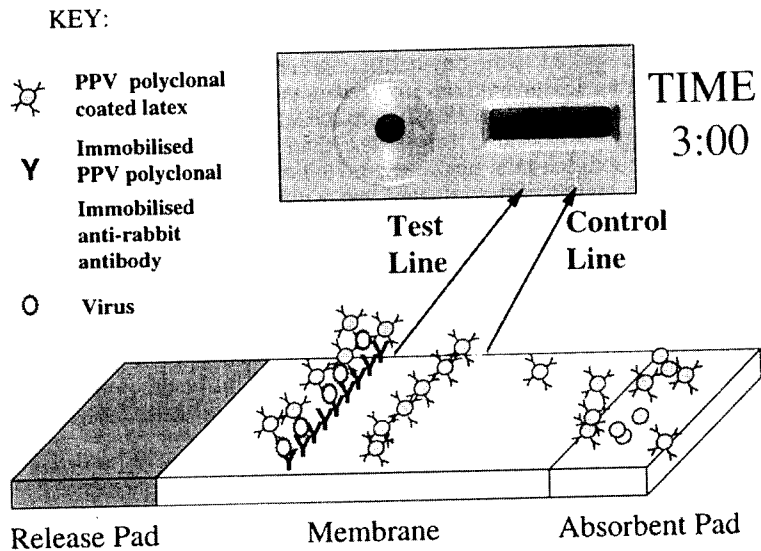
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Tables

1. Summary of PPV test results from plum samples received at CSL from 1998 onwards (until June, 2000)

ELISA results		RT-PCR results		RFLP analysis		
+ve	-ve	+ve	-ve	D-strain	M-strain	C-strain
11	2523	11	0	11	0	0

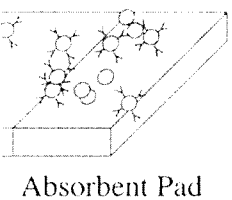
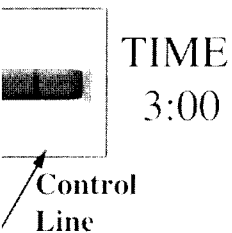
Figures



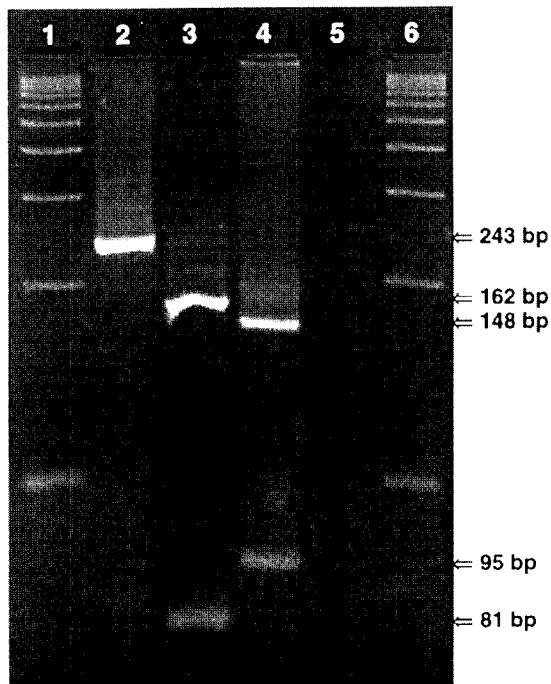
1. A schematic diagram showing the detection system employed by the one-step PPV LFD.

ed at CSL from 1998 onwards

RFLP analysis		
in	M-strain	C-strain
	0	0



mployed by the one-step PPV



2. Detection and strain typing of PPV by RT-PCR and subsequent RFLP analysis. Agarose gel (4% Metaphor-TBE) showing a D-strain PPV positive sample, with PCR products either uncut (lane 2) or cut with *Rsa* I (lane 3) or *Alu* I (lane 4). RT-PCR from uninfected *Prunus* control is shown in lane 5. Lanes 1+6 contain 100 bp ladder (AB gene).

Sample:

Healthy leaves
Infected leaves

Healthy leaves	10	9	8	7
Infected leaves	0	1	2	3

3. Detection of PPV using the one-step LFD. The sensitivity of the device is demonstrating by using sap extracted from samples of ten leaves, which include varying numbers of PPV-infected leaves, from 0 (a healthy sample) to 3 (a moderately infected sample).