Session 2

Diagnostics for Viruses, Phytoplasmas and GMOs

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VIRUS SPECIFIC ANTIBODIES FROM A PHAGE-DISPLAY LIBRARY

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ABSTRACT

Antibody fragments (scFv) that bind specifically to particles of cucumber mosaic cucumovirus (CMV), and potato leafroll luteovirus (PLRV) were obtained from a library which encodes a diverse array of synthetic antibody fragments displayed on the surface of filamentous bacteriophage. Several virus-specific clones were obtained after only three or four rounds of selection against particles of CMV or PLRV. The nucleotide sequences of the secreted scFv were typical of immunoglobulin variable domains and both phage-displayed and soluble scFv reacted with either CMV- or PLRV-infected plant sap extracts. In immunoblotting tests, soluble scFv preparations reacted with SDS-denatured coat protein extracted from purified preparations of CMV isolates belonging to either subgroups I or II, and also with protein extracted by SDS-treatment of seeds harvested from naturally infected lupin plants. The PLRV specific scFv were sub-cloned into an expression vector so that they were obtained fused to alkaline phosphatase (AP). The scFv-AP fusions were functional, detecting PLRV in infected sap. The results demonstrate the feasibility, and potential applicability, of recombinant antibodies to the detection of plant viruses.

INTRODUCTION

The use of polyclonal and monoclonal antibodies in plant virology has provided useful knowledge and insight into the processes of infection in plants, and of transmission by vectors, as well as becoming the cornerstone of the detection and diagnosis of virus diseases (Torrance, 1995). It has been shown that functional fragments of antibody molecules can be expressed, fused to the minor coat protein pIII, on the surface of filamentous phage (phage display; McCafferty et al., 1990; Hoogenboom and Winter, 1992). These fragments comprise the heavy and light chain variable (antigen binding) domains of the antibody molecules linked by 15 amino acids to form a single polypeptide chain (scFv). Specific scFv can be obtained from a population of phages carrying many different scFv by binding to and then eluting from antigen. The selected phage preparation can then be enriched for binders by re-infecting *E. coli* with the eluted phage and repeating the procedure. In this way genetically pure populations of phage which encode the scFv can be obtained after several repeated cycles. Large combinatorial phage display libraries have been produced containing $>10^8$ different clones and it has been shown that a range of recombinant immune reagents with diverse specificities can be isolated from such libraries (Nissim, et al., 1994).

Selection from phage display libraries yields specific antibody fragments without the need either to immunize animals or to use hybridoma technology, and should therefore overcome several difficulties in the production of conventional polyclonal sera or monoclonal antibodies such as poor immunogenicity, toxicity of the antigens or high production costs. However, it is not clear whether such reagents would be of use for plant virus detection and diagnosis, and this paper describes some work to investigate the potential applicability for

plant viruses.

MATERIALS AND METHODS

The methods used were those described by Ziegler et al., 1995. The scFv were obtained from the MRC human synthetic scFv library (Nissim et al., 1994). The scFv are fused to the minor fd phage coat protein pIII, an amber mutation between the sequences encoding the scFv and pIII means that strains of *E. coli* that do not suppress the amber stop codon (non-suppressor strains) secrete soluble scFv. The scFv have a 15 residue peptide (myc-tag) attached to the C terminus which provides a means of detection and purification of soluble scFv using myc-tag-specific monoclonal antibody 9E10.

The library stock was amplified and particles were rescued by superinfection with helper phage M13VCS, which supplies the viral genes to permit packaging of the phagemid DNA and the display of the scFv-pIII fusion proteins on phage. For each round of selection, immunotubes (Nunc) were coated by incubation with a purified preparation of CMV or PLRV 10-50 $\mu g.ml^{-1}$ in 50mM carbonate (coating) buffer pH 9.6 or phosphate-buffered saline (PBS) respectively, overnight at 4° C. The tubes were then rinsed with PBS and blocked with 2% non-fat dried milk (Marvel) in PBS. Phage preparations containing 10^{12} cfu were added to the tubes and incubated for 4 h at room temperature (c. 22°C). After extensive washing, by rinsing the tubes 20 times with PBS containing 0.05% Tween 20 and 20 times with PBS, bound phage was eluted with 100 mM trimethylamine and then used to infect *E.coli* cells. Phage particles were rescued by superinfection with helper phage.

The phage-displayed antibody and the preparations of soluble scFv obtained either as periplasmic extracts or culture supernatants were tested for binding to CMV by PTA-ELISA (Torrance, 1992). Microtitre plates containing extracts of CMV strain Fny (subgroup I) infected *Nicotiana clevelandii* and *N. glutinosa* plants (sap dilution $g.\mu l^{-1}$ in coating buffer) were incubated 16 h at 4 °C, then rinsed and blocked as above. The scFv preparations were mixed 1:10 with monoclonal antibody 9E10 and incubated for 3 h at room temperature. The plates were rinsed and incubated with anti-mouse (or anti-rabbit) alkaline phosphatase conjugate (Sigma Immunochemicals) as appropriate followed by substrate. The same method was used for PLRV specific scFv except that preparations of PLRV ($1\mu g.ml^{-1}$) were used to coat microtitre plates. In addition a direct ELISA was done using polyclonal anti-PLRV antibodies as coating antibodies and scFv-AP fusions or polyclonal-AP conjugate as detecting antibodies.

RESULTS AND DISCUSSION

Several clones secreting specific scFv were obtained after selection on CMV particles. One of the scFv was tested for cross-reactivity with CMV and other small icosahedral viruses; it did not react with plant extracts containing tomato aspermy virus (TAV) or tobacco necrosis virus (TNV) (Table 1). In the same test homologous polyclonal anti-CMV antibodies gave a mean absorbance value of 1.8 with CMV-Fny. The scFv reacted with SDS-denatured coat protein of CMV strains Fny (subgroup I) , LW and LS (subgroup II) in immunoblots (Ziegler et al., 1995) and also with CMV coat protein in naturally infected lupin seed extracts. There was no reaction with extracts from non-infected seeds.

Table 1. Absorbance values (A_{405nm}) obtained in PTA-ELISA of cucumber mosaic virus (CMV) , tomato aspermy virus (TAV) and tobacco necrosis virus (TNV) isolates using scFv recombinant antibody.

scFv* (PE dilution)	Virus isolate CMV-Fny	TAV	TNV	Non-infected Plant extract
1:2	0.43**	0.06	0.09	0.09
1:5	0.21	0.13	0.1	0.08
1:10	0.15	0.08	0.1	0.07

* scFv preparation was a bacterial periplasmic extract (PE)
** Absorbance value (A_{405nm})

The properties of scFv secreted by four clones obtained by selection on PLRV particles were studied. Phage-displayed scFv reacted with PLRV infected sap extracts, for example scFv 4.10 gave an absorbance value of 1.27 (control 0.13) after 4 h substrate incubation. The scFv were sub-cloned to produce alkaline phosphatase fusion proteins and tested in ELISA. The fusion proteins detected PLRV in extracts of infected *Physalis floridana* leaves (Table 2) and further binding studies are in progress.

Table 2. Absorbance values (A405nm*) obtained in ELISA of potato leafroll infected *Physalis floridana* sap extracts using scFv-alkaline phosphatase fusion proteins.

Dilution scFv **	sap dilution (reciprocal) 4	8	16	32	negative control
1/10	0.70	0.44	0.32	0.18	0.15
1/100	0.31	0.24	0.21	0.13	0.10
1/1000	0.27	0.22	0.19	0.12	0.09

* A405nm after overnight incubation of substrate
** dilutions of scFv periplasmic extract

The results reported here show that virus-specific scFv were obtained quickly (within four weeks), and without recourse to animal immunisations, from a synthetic phage display library. Although the absorbance values obtained in ELISA with the scFv specific for CMV were weaker than those with the polyclonal antibody preparation, the scFv were used without any modification after only four rounds of selection. ScFv are monovalent molecules and it is possible to produce bivalent molecules by shortening the linker peptide which should increase the avidity (Holliger et al., 1993) and therefore improve performance in ELISA. The results with PLRV specific scFv show that it is possible to express functional scFv fusions with alkaline phosphatase in bacteria.

Our work has demonstrated the feasibility of using recombinant antibody methods to detect plant viruses. However, further work is needed to improve avidity of the scFv and to devise ELISA methods based completely on bacterially expressed antibody fragments. We think

that further development of these reagents is worthwhile because bacterial expression of antibody-like proteins for the detection and diagnosis of plant pathogens would produce standardised, reproducible assays at a fraction of the costs of production of monoclonal antibodies. Furthermore, costly methods for storage of hybridoma cell lines would be avoided (an advantage in countries where supplies of liquid nitrogen are unreliable).

ACKNOWLEDGEMENTS

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MONITORING RELEASES OF TRANSGENIC PLANTS: THEORETICAL AND PRACTICAL CONSIDERATIONS

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ABSTRACT

Regulatory requirements prescribe monitoring of transgenic releases in order to check on the spread and eventual fate of the released organisms and to monitor gene flow. In many cases the phenotypic markers used in the development of a transgenic plant may not be suitable for large scale reliable non-destructive monitoring e.g. enzyme assays for antibiotic resistance or biochemical assays for altered physiological properties. We have therefore evaluated the use of DNA methods and in particular the polymerase chain reaction (PCR) for the detection of transgenic plants either from individual samples or from bulk samples. We have assessed factors that might contribute to inhibition of the assay and the level of accuracy and feasibility of this apporach in the monitoring of large scale releases of transgenic plants.

INTRODUCTION

Recent years have seen a rapid increase in field releases of transgenic plants (Ahl Goy *et al.*, 1994) and the launch of products from this technology onto the US and European markets. Genetic modification (GM) provides an enormous potential for tackling the growing demand both for food and raw materials. However there has been an ongoing controversy concerning the safety of transgenic releases and the possibility that the transfer of transgenes to natural populations might be deleterious to the natural or agricultural environment (reviewed in Rogers and Parkes, 1995). These concerns have been tackled by legislation both in the UK and elsewhere ensuring that at all planned releases of transgenic plants are assessed for their safety before permission is granted for the release and that monitoring programmes are carried out. The initial purpose of the monitoring excercise was to prevent any escape of the transgene from the release site. In early, small-scale, experimental releases 'monitoring' was achieved by destruction of the GM plants at the end of the experiment. Plants belonging to the same species as the GM crop growing in adjacent areas to the release were also destroyed. However as our knowledge on the spread of transgenes, their stability, and their effects has increased, this approach has been deemed unnecessary and legislation has been adapted accordingly. Monitoring has in fact played an important part in the evolution of legislation in this area by improving the definition of necessary isolation distances between transgenic crops and

non-transgenic crops or compatible native species, assessing the efficacy of barrier rows of non-transgenic plants and evaluating the need for preventing the flowering of the transgenic crop (Muench, 1990). Thus future monitoring exercises may have a different role, namely to provide information on the spread of transgenes and only intervene should very clear adverse effects become obvious.

In order to monitor the spread of transgenes effectively from large scale releases and field samples, methods need to be adopted which are rapid, accurate, and with potential for quantitation. Accuracy is particularly important when measuring rare events. In this case false positives can have large effects on the final result. For example Karieva *et al.* (1994) found that detection of false positives at more than one in 10 000 led to substantial over-estimates of transgene dispersal. Speed of assay is also an important factor since large numbers of samples need to be assayed in order to detect dispersal rates which, for example, in an experiment with transgenic oil seed rape was found to be 0.00033% at 47m from the crop (Scheffler *et al.*, 1993).

We have evaluated the use of the polymerase chain reaction (PCR) for the detection of transgenes in two important UK crops and their wild relatives considering factors such as speed of assay, reliability and possible inhibitory factors present in field samples.

MATERIALS AND METHODS

Plant material

Non transgenic material was either grown in growth cabinets or field collected. Transgenic sugar beet was obtained from Dr MJ Crawley (Silwood Park, Imperial College, London), transgenic oilseed rape was obtained from (Dr. J Sweet, National Institute of Agricultural Botany)

DNA extraction methods

Large scale DNA extraction was essentially as described in Rogers and Lonsdale (1992). Small scale DNA extraction was carried out either as described by Wang *et al.* (1993) or Edwards *et al.* (1991)

PCR amplification

Amplification was carried out in a Perkin Elmer 9600, or Hybaid Omni machine in 25μl reaction volumes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.001% (w/v) gelatin, 1.25 mM of each dNTP, 0.63 units of AmpliTaq (Perkin Elmer) Taq DNA polymerase, 100 ng each of primers Amplification conditions were as follows: one cycle of {94 °C 1 min, 50 °C 30 sec, 72 °C 3 min} followed by 28 cycles of {94 °C 30 sec, 50 °C 30 sec, 72 °C 3 min} and one cycle of {94 °C 30 sec, 50 °C 30 sec, 72 °C 10 min}. 20 μl ot the PCR products were analysed by agarose gel electrophoresis in 1.5% agarose/TBE (Sambrook *et al.*, 1989).

RESULTS

Sensitivity of PCR reaction on plant material from different tissues and sources

A comparison was made of different oil seed rape tissues to investigate whether the source of the tissue affected either the sensitivity of the PCR reaction, and/or whether the quality of the DNA in terms of PCR inhibition was affected. Table 1 indicates that although the source of DNA affected the maximum amount of DNA that could be used in a PCR reaction the sensitivity of the assay remained constant.

Table 1. Upper and lower limits of oil seed rape DNA in PCR reactions.

Tissue	upper limit of DNA in the PCR reaction	lower limit of DNA in the PCR reaction
Leaf	400 ng	10pg
Seed	4 ng	10pg
Root	0.4 ng	10pg

Investigation into inhibition of the PCR reaction

One drawback of PCR as an assay is that it is known to be subject to inhibition by a variety of natural and chemical compounds. Current agricultural practices make use of a wide range of agrochemicals and it is likely that samples collected from the field might contain residues of such chemicals, particularly if samples are collected soon after treatments. The effect of a range of such chemicals on the PCR reaction was therefore tested. 100 ppm of each compound was added to leaf samples before extraction. This concentration was chosen to reflect maximum levels likely to be found in the field. Results are presented in Table 2

Specificity of the PCR reaction

In order to assess whether the PCR primers selected were specific for the transgene, non-transgenic oil seed rape, a wild relative of oil seed rape: charlock and a cultivated relative cabbage were also tested. DNA was extracted from leaf material from these three species and PCR amplification was performed as above. No product was obtained for any of these species (result not shown) indicating that the specificity of these primers is suitable for detecting the spread of this transgene to wild and cultivated relatives of oil seed rape.

Table 2. Effect of agrochemicals on the PCR reaction

Use	Target crop	Chemical	PCR result
insecticide	potato	aldicarb	+
insecticide	potato	phorate	+
insecticide	potato	pirimicarb	+
insecticide	sugar beet	fenvalerate	+
insecticide	sugar beet/OSR	cypermethrin	+
fungicide	OSR	carbendazim	+
fungicide	OSR	iprodione	+
fungicide	OSR	prochloraz	+
dessicant	OSR	glyphosate	+
herbicide	potato	paraquat	+
herbicide	OSR	propyzamide	+
slug pellets	sugar beet	methiocarb	+

Individual assays on field samples of transgenic sugar beet

PCR was assessed in comparison to an enzyme assay for individual sampling in an experimental field release experiment using transgenic oil sugar beet. The purpose of this experiment was to test the ease of sampling, reliability and speed of these two methods for discriminating between transgenic and non-transgenic plants. In this experiment the plants were a mixture of transgenic and non-transgenic plants. Two PCR targets were used; the *nptII* gene which provides resistance to the antibiotic kanamycin and beta-glucuronidase (GUS), a widely used plant reporter gene. Results from this experiment are presented in Table 3

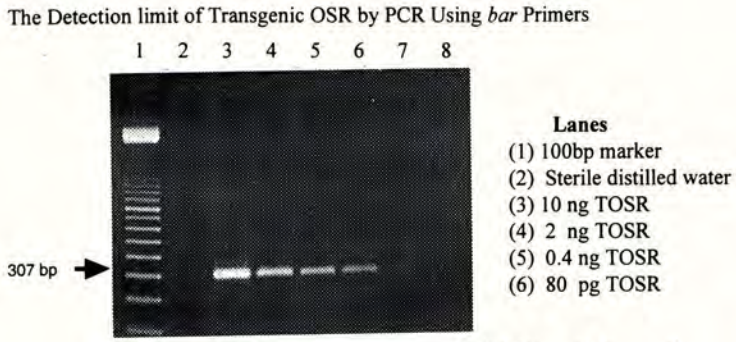
Table 3 Results of an evaluation of PCR as an assay for transgenic plants in comparison to an enzyme assay.

Assay	No. of plants tested	Positives	Negatives	Speed of assay/sample
PCR/*nptII* and GUS targets	53	26	27	30 min for DNA extraction, 5 min for PCR set up, 3 hrs for PCR reaction, 1 hr for analysis of PCR results
PCR control	53	53	0	
GUS enzyme assay	51	26	25	1.2 hr

Bulked assays on oil seed rape samples.

Since extraction from individual samples is time consuming, the alternative of extracting from a mixed bulk sample was assessed. Initial experiments used DNA extracted from transgenic and non transgenic samples to assess the sensitivity of the assay. A dilution series was made of DNA extracted using a standard DNA extraction method and dilutions were tested by PCR using specific PCR primers for the herbicide tolerance gene *bar*. Fig 1 shows that the minimum DNA concentration detectable in this reaction was 80 pg of total genomic DNA. This sets the minimum sensitivity limit for this DNA extraction method, oil seed rape leaf material and a single copy gene target.

Figure 1. Dilution series of DNA illustrating sensitivity of PCR assay



The experiment was repeated using 80pg of the transgenic DNA and increasing amounts of non-transgenic DNA. Since large amounts of DNA were found to inhibit the PCR reaction, this experiment was necessary in order to determine the number of plants which can be bulked and still allow detection of a single transgenic plant. The maximum ratio which gave a positive PCR reaction was found to be 1:1000 (results not shown) indicating that bulk samples of up to 1000 plants could be used.

DISCUSSION

We have investigated the use of PCR as an assay for the detection of transgenic plants by evaluating various factors which would affect this method on real field samples. Since in a monitoring excercise it may be necessary to use a variety of plant tissues such as leaf, root and seed samples, the effect of material source on the sensitivity of the assay was assessed. It was found that although the bottom limit of detection was unaffected, the maximum levels of DNA differed. DNA extracted from leaf was found to be the least inhibitory to the reaction. This effect is probably due to dilution of inhibitory factors in the lower limit samples, and differences in the abundance of PCR-inhibitory substances in the DNA from different plant tissues. Similar observations have been made e.g. using DNA extracted from potato tuber which is found to be particularly inhibitory to PCR (HJ Rogers unpublished results). The inhibitory effect of a range of agrochemicals was also tested. None of those tested appeared to show significant inhibition of the PCR reaction.

This indicates that the use of field grown material in the assay should not present a problem, even if the crop has been recently sprayed. Monitoring also requires an assessment of the gene flow of transgenes from the GM crop to related species. The PCR primers targetted to a widely used herbicide resistance gene were tested on charlock (*Sinapis arvensis*) and cabbage. No signal was detected indicating that these primers are suitable for field monitoring of gene flow.

PCR assays on individual leaf samples were used to differentiate between field grown transgenic and non-transgenic sugar beet. Although the PCR reaction was as expected found to be reliable, the speed of the assay was considerably longer than an enzyme assay used for comparison. However suitable enzyme assays are not always available, and the time required for the PCR assay could be reduced by using a faster DNA extraction method, and improvements in the speed of the PCR reaction. Automation of the DNA extraction and PCR set up would also considerable reduce the hands-on time needed.

As an alternative bulk assays were assessed. It was found that samples from up to 1000 plants could be safely pooled thus reducing drastically the number of DNA extractions and PCR reactions to be performed. This seems a valuable option when field data on the extent of transgene dispersal is required without identification of individual transgenic plants. We are currently developing methods to assist in the quantitation of this bulk assay, and will be evaluating its use on the first commercial release of oil seed rape in the UK.

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DETECTION OF BANANA STREAK VIRUS

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ABSTRACT

Banana streak virus (BSV) a member of the Badnavirus group, causes severe problems in banana cultivation, reducing fruit yield, restricting plant breeding and the movement of germplasm. Current detection methods are relatively insensitive. A PCR based diagnostic method has been developed that has been shown to be both reliable and sensitive for the detection of BSV in infected banana plants in Nigeria.

INTRODUCTION

Banana is an important crop for smallhold farmers of west and central Africa providing both a substantial food and cash source. Currently, improvements in the disease resistance, fruit yield and other agronomic qualities of bananas and plantains are being investigated by the International Institute for Tropical Agriculture (IITA), Nigeria. These improvements are hampered by the recent diagnosis of banana streak virus (BSV) in the Banana breeding stocks. Quarantine regulations prevent the movement of infected plant materials, even improved varieties.

BSV is a member of the badnavirus group (Lockhart, 1990), non-enveloped bacilliform particles of size 30 x 130-150 nm, possessing a double-stranded DNA genome of 7.4-8.0 kB. There is now a reasonable body of information on these viruses with the sequences of four reported.(Medberry *et al.*, 1990;Hay *et al.*, 1991;Bouhida *et al.*, 1993;Hagen *et al.*, 1993) and known for at least two others. This information has revealed common features of the group. They possess regions of homology to an RNA binding domain (RB), aspartic protease (AP), reverse transcriptase (RT) and an RNase H (RH). These features together with a potential $tRNA^{met}$ binding site, suggests they are pararetroviruses (Medberry *et al.*,1990). The use of RT in their replication can potentially lead to high degree of variation between isolates and different group members, and such variation has already been reported (Lockhart, 1993). Functions for other genes have also been postulated.

Current methods of detection are by characteristic symptoms and/or serological methods, both of which are relatively insensitive. A sensitive and reliable method of detection is required for BSV, in the first instance for the banana germplasm of Nigeria, and later to account for any source of germplasm.This method should be sensitive to allow for the detection of a low-titre virus in tissue culture plantlets and reliable. In addition it should be able to accomodate the variation likely to be found

in different samples. It has already been shown that the virus is serologically heterogenous (Lockhart and Olszewski, 1993). A PCR based approach could fulfil these conditions. Badnavirus sequence information currently available allows conserved regions to be identified. Degenerate primers covering these regions have previously been used in badnavirus detection (Lockhart and Olszewski, 1993, Braithwaite *et al.*, 1995). These primers did not amplify appropriately sized bands from BSV-infected Nigerian banana plants. It was decided that the sequence of a Nigerian isolate of BSV should be used to design PCR primers for the detection of Nigerian BSV.

MATERIALS AND METHODS

Virus isolation and purification

BSV-infected banana plants were maintained in the greenhouse. Leaf was finely ground in liquid nitrogen and blended into 2 volumes buffer A (50 mM sodium phosphate pH 6.1, 5mM DTT, 5mM DIECA, 0.5% PEG 6000). Celluclast (Novo Nordisk) was added to 2%, incubated with stirring at 37°C for 2 h then overnight at room temperature. Triton X-100 was added to 1% and the incubation carried out for a further 30 min. All subsequent steps were carried out at 4°C. After low speed centrifugation, 10,000g for 10 min, the supernatent was centrifuged at 120,000g for 90 min. The pellet was resuspended in 100 ml buffer A, and centrifuged through a 5% sucrose cushion at 120,000g for 2.5 h. The pellet was resuspended in 5 ml buffer A. The virus was further purified by centrifugation in a 0-40% $CsSO_4$ gradient in 10% sucrose [steps 40,30,20,10,0] at 120,000g for 2.5 h. The viral band was identified by EM, diluted four fold in buffer A, pelleted at 150,000g for 60 min and resuspended in 100μl 50 mM sodium phosphate pH 6.1. ISEM was carried out (by B.Lockhart) according to Lockhart (1986).

Cloning and sequence analysis

Virion DNA was purified by digestion of virus particles with Proteinase K at 1mg/ml in 100mM TrisCl pH 8.0, 2mM $CaCl_2$, 2% SDS, for 2 h at 65°C. After phenol extraction the DNA was precipitated, washed in 70% ethanol, dried and resuspended in 50 μl TE. The DNA was digested with *Eco*R1 and the resulting fragments cloned into pBluescript II SK+ (Stratagene). A clone was identified as a badnavirus by sequence homology. Using this sequence information primers were designed to allow PCR amplification of the entire virion DNA. The 7.5 kb product was automatically sequenced using an ABI sequencer and Prism system. The sequence was analysed using the GCG sequence package (Devereux *et al.*, 1984).

Primer design and PCR

PCR primer pairs were chosen from aligned amino acid sequences corresponding to the aspartic protease and reverse transcriptase regions of the derived BSV sequences. The PCR was performed on DNA isolated from Banana leaves using the method of Li *et al.*, (1994), using the basic protocol described by manufacturers of the *Taq* DNA polymerase (Gibco BRL). The conditions were 94°C for 2 min, [94°C for 1 min,40-50°C for 1 min, 72°C for 1min] x 30 cycles, followed by a 5 min extension at 72°C. Reaction products were separated by electrophoresis through a 1.5% agarose gel and detected by fluorescence of ethidium bromide under UV light. Following photography, Southern blotting was carried out, and the resulting

membrane probed with a ^{32}P-labelled full-length BSV PCR product.

RESULTS

Virus purification

The virus purification yielded particles of the expected size and shape although the yield was low. They could be trapped by BSV antiserum coated carbon grids. The virus could be detected by ISEM in a crude extract of infected leaf, but not in leaf-dip preparations.

Virus sequence, homology and primer design

The complete 7454bp nucleotide sequence of both strands of BSV-Nigerian isolate was determined (Harper and Hull, manuscript in preparation). The + strand contains three large ORF's. This number, their size and order are similar to other badnavirus sequences. The sequence is sufficiently different from other badnaviruses for it to be considered a distinct virus. The BSV ORF III shows ~60% similarity overall to the ORF III sequences of other badnaviruses, with regions of higher similarity particularly over the putative aspartic protease and reverse transcriptase coding regions. PCR primer sequences were selected from within these coding sequences.

PCR

After using a number of different primer sets, one pair consistently gave a strong amplification product, of the expected size of 664bp. Hybridisation with a BSV probe and sequencing of this product confirmed its origin as BSV. Amplification of this band could also be achieved from crude preparations from infected plants, although inhibitors of the PCR reaction caused some problems.

Comparison of PCR and ISEM

A controlled blind trial of PCR and ISEM as BSV detection methods was carried out. Crude viral preparations from a number of banana cultivars were sent to two different laboratories for either PCR or ISEM analysis. The results are shown in Table 1. They show that ISEM could detect BSV in 9/36 samples, and PCR could detect BSV in 32/34 of the samples. ISEM did not detect BSV in any sample that was not also detectable by PCR. PCR is clearly more sensitive a technique than is ISEM.

DISCUSSION

The virus isolated from symptomatic banana plants has been demonstrated to be a badnavirus by morphology and both sequence and serological homology to other badnaviruses. Using the sequence information a diagnostic PCR assay has been developed that allows highly sensitive and specific detection of BSV in Nigerian banana plants.

The apparently high degree of infection of banana by BSV shown in this study, is mimicked by the widespread infection of sugarcane by the related sugarcane bacilliform badnavirus (SCBV, Comstock and Lockhart, 1990). In this case, the virus isolated from Nigerian plants appears to have low variability as judged by restriction

Table 1. Comparative detection of BSV by symptom, ISEM and PCR.

Sample No	Symptoms	ISEM RESULT	PCR result
1a	Symptomless	-ve	+ve
1b	Necrotic	+ve	+ve
1c	Symptomless	-ve	+ve
1d	Necrotic	-ve	+ve
2	Symptomless	-ve	-ve
3	Symptomless	-ve	+ve
4	Symptomless	+ve	+ve
5a	Necrotic	-ve	+ve
5b	Symptomless	+ve	+ve
5c	Symptomless	+ve	+ve
6a	Symptomless	+ve	+ve
6b	Necrotic	-ve	+ve
7a	Necrotic	+ve	+ve
7b	Necrotic	+ve	+ve
7c	Chlorotic	+ve	+ve
7d	Chlorotic	+ve	+ve
8	Symptomless	-ve	-ve
9	Symptomless	-ve	
10	Symptomless	-ve	
11a	Symptomless	-ve	+ve
11b	Symptomless	-ve	+ve
11c	Symptomless	-ve	+ve
12a	Chlorotic	-ve	+ve
12b	Necrotic	-ve	+ve
12c	Symptomless	-ve	+ve
13a	Symptomless	-ve	+ve
13b	Symptomless	-ve	+ve
14a	Necrotic	-ve	+ve
14b	Symptomless	-ve	+ve
14c	Symptomless	-ve	+ve
15	Symptomless	-ve	+ve
16	Necrotic	-ve	+ve
17	Necrotic	-ve	+ve
18	Symptomless	-ve	+ve
19	Symptomless	-ve	+ve
20	Chlorotic	+ve	+ve

length polymorphism analysis (data not shown).

As shown above, PCR is more sensitive than current ISEM techniques in the detection of BSV. Similar PCR-based assays have been employed for the detection of rice tungro bacilliform virus (RTBV, Takahashi *et al.*,1993) and SCBV (Braithwaite *et al*, 1995), and in both cases PCR was judged to be more sensitive than serological methods. The method has proved sufficiently sensitive to be used on banana

plantlets in tissue culture, allowing early elimination of infected germ plasm. In this case, PCR is used as a first stage screen and PCR-negative plants are subsequently tested by EM analysis of virus preparations. The current approach was designed to detect BSV in Nigerian banana plants; whether BSV from other regions can also be detected is currently being investigated.

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LABORATORY METHODS FOR VIRUS DETECTION IN FRUIT TREES

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ABSTRACT

Reliable laboratory methods are urgently needed for the detection of viruses in fruit trees to replace the graft tests currently required to monitor plants of the highest health status. These tests are costly and take a long time to complete and we report on the effectiveness of PCR and non-isotopic hybridisation assays for several fruit tree viruses in comparison with ELISA. Immuno-capture (IC)-PCR, detected a broad spectrum of apple chlorotic leaf spot virus (ACLSV) isolates and was superior to ELISA in detecting the virus in samples taken throughout the growing season from two apple trees, a plum and a quince tree. The 358 nt PCR product of an isolate from plum was cloned and used to prepare digoxigenin-labeled transcripts. In dot-blot hybridisation tests, these detected the virus in extracts from *Chenopodium quinoa* but not in extracts from woody plants. Primer pairs were designed to conserved regions in the 3.5 kb at the 3' end of the genome of apple stem grooving virus (ASGV). The pair that were best for detection of UK isolates of ASGV in *C. quinoa* were used to detect the virus in fruit trees; however, detection by PCR was poor in comparison to ELISA using commercially available reagents. By contrast, prune dwarf virus (PDV) was successfully detected by ELISA, IC-PCR and hybridisation (digoxigenin-labeled cRNA probe derived from cloning the 172 bp PCR product) in extracts from infected cherry and plum in the spring but IC-PCR and hybridisation were both more successful than ELISA for detection later in the growing season.

INTRODUCTION

Viruses infecting fruit trees frequently cause few symptoms although they adversely affect fruit quality and/or productivity (Campbell & Sparks, 1986; Cameron, 1977; Cropley & Posnette, 1973). The subtle nature of symptoms and the scope for transmission and dissemination through grafting and propagation led in the past to an accumulation of viruses. Many cultivars became infected throughout with several viruses so that when they were tested using the range of indicator plants available in the 1950s and 1960s no uninfected source plants could be found (Cropley, 1968). The advent of heat therapy for virus elimination (Kassanis, 1950) led to the production of virus-free material world-wide (Nyland & Goheen, 1969) and its distribution to growers through Certification schemes designed to ensure freedom from pathogens and trueness-to-type. Efforts are now being made to standardise the methods used for testing fruit trees to increase confidence in trade throughout the EC (EPPO, 1992). The highest grade of material (designated Virus Free) requires testing on woody indicator plants but this is time consuming (some tests take 3yr to complete), needs a high degree of skill and is very expensive (about 35 trees are in the

ground for 3 to 6 yr to fully test one apple tree). If schemes to produce healthy planting material are to survive, it is essential to replace these tests with quick, convenient and reliable laboratory assays. This paper describes progress in developing and/or assessing the reliability of protocols for assaying three important fruit tree viruses (apple chlorotic leaf spot - ACLSV, apple stem grooving virus -ASGV and prune dwarf virus - PDV) by three types of test: ELISA, non-isotopic hybridisation and immunocapture polymerase chain reaction (IC-PCR).

MATERIALS AND METHODS

Tissue sampling

Monthly tests were conducted on samples from the current year's growth on five shoots from seperate limbs of each tree as follows. ACLSV: two apple trees cv. Golden Delicious (12/29, 12/17, Old Museum, HRI East Malling), one Myrobalan plum (HRI-EM), one quince cv. Serbian (National Fruit Collection (NFT), Brogdale, Kent); ASGV: two apple trees cv. Wijcik, New Museum and cv. Golden Delicious 12/29 Old Museum, both HRI-EM and two oriental pear trees 2/26, 4/17, both from NFT; PDV: two cherry trees and two plum trees (2/7, 2/30, 4/4, 4/6 respectively, all Old Museum, HRI-EM). Samples were tested immediately or stored for up to one week at 4^0C before processing.

Preparation of extracts

All extracts were prepared by grinding 0.1 g tissue in 2 ml pre-cooled buffer and diluting immediately to the appropriate concentration for the test (as determined in preliminary experiments). Positive and negative controls were prepared similarly and included in all tests.

PDV: extracts for ELISA were diluted to 1:100 (wt:vol.) in phosphate buffered saline (PBS) containing 0.5 ml/litre Tween 20 (PBS/Tween), 20 g/litre polyvinylpyrrolidone (mol. wt 44000) and 2 g/litre ovalbumin (PVP/ov). PCR samples were diluted to 1:500 in the same buffer and clarified in a microcentrifuge at 13 500 rev/min for 2 min. Samples for hybridisation were diluted to 1:500 in buffer containing 200 mM dibasic potassium phosphate, 10 mM sodium diethyldithiocarbamate (DIECA), 5 mM DL-dithiothreitol (DTT), 0.1% Triton X-100.

ACLSV: extracts for ELISA were diluted to 1:100 in Tris/Tween/PVP (10 mM Tris-HCl pH 7.6, 10 mM magnesium sulphate, 0.05% Tween 20, 2% PVP); PCR samples were diluted in the same buffer to 1:100 for apple and 1:500 for plum and pear leaves and clarified by centrifugation. Samples for hybridisation were extracted as for PDV.

ASGV: extracts for ELISA were diluted in PBS/Tween/PVP/ov to 1:20 and for PCR in the same buffer to 1:100.

ELISA

The $F(ab')_2$ indirect ELISA system (Barbara & Clark, 1982) was used for PDV and ACLSV

using reagents prepared by standard procedures from antisera made at HRI East Malling. A commercial ELISA kit (Loewe Biochemica GmbH, Germany) was used for the detection of ASGV according to the manufacturer's instructions. Test and control extracts were added to duplicate wells of pre-coated polystyrene micrototre plates (Nunc-ImmunoPlate Maxisorb F96, NUNC, Roskilde, Denmark) and incubated overnight at 4°C. Absorbance values at A_{450} (PDV and ACLSV assays) or A_{405} (ASGV assay) were recorded with a BioRad Model 3550 Microplate Reader. Test samples were regarded as positive when their mean absorbance values were more than twice the mean values of the negative controls in the same plate.

Immuno-capture PCR

PDV: PCR microtitre plates (Omniplate 96, Hybaid) were coated with virus-specific $F(ab')_2$ fragments in sodium carbonate coating buffer (100 µl/well) and incubated for 5 hours at 30°C. Excess antibody was removed by adding 120 µl/well of PBS/Tween, incubating for 2 min at room temperature and decanting.

The washing step was repeated once. Sample extracts were added (50 µl/well) to duplicate wells and incubated overnight at 4°C. Extracts were removed by pipette, 100 µl/well PBS/Tween added, incubated for 2 min and removed. The washing step was repeated twice and followed by a rinse of 100 µl/well with water. These washes were removed by pipette, rather than by decanting, to avoid cross-contamination between samples. PCR was performed as a one-tube protocol in a 25 µl volume containing 20 mM Tris-HCl pH 8.4, 50 mM KCL, 1.5 mM $MgCl_2$, 0.3% Triton X-100, 0.25 mM each dNTP, 25 pmole each primer, 0.25 units AMV Reverse Transcriptase (GIBCO/BRL) and 0.5 units Taq DNA Polymerase (GIBCO/BRL). Reactions were overlaid with 50 µl mineral oil and incubated at 42°C for 45 min and 92°C for 2 min followed by 40 cycles of 92°C for 30 s, 60°C for 30 s, 72°C for 1 min. The PDV primers were designed from the data of Bachmann *et al.* (1994) as used by Parakh *et al.* (1995): PDV1 (5' ATG GAT GCG ATG GAT AAA ATA GT 3; corresponding to nucleotides 1838-1860) and PDV2 (5' TAG TGC AGG TTA ACC AAA AGG AT 3'; corresponding to the reverse complement of nucleotides 1988-2010) amplifying a 172 bp product at the 3' end of PDV RNA-3.

ACLSV: TreffLab 0.5 ml microfuge tubes (Scotlab) were coated with virus-specific immunoglobulins, as above and incubated for 3 h at 30°C. The procedure was as for PDV except that the wash buffer was Tris/Tween and the PCR annealing temperature was 50°C. The primers were those of Candresse *et al.* (1995): ACLSV53 (5' GGC AAC CCT GGA ACA GA 3'; corresponding to nucleotides 6876-6892) and ACLSV52 (5' CAG ACC CTT ATT GAA GTC GAA 3'; corresponding to the reverse complement of nucleotides 7213-7233) amplifying a 358 bp PCR product within the coding region of the viral coat protein gene (German *et al.*, 1990).

ASGV: five primer pairs were designed to regions conserved between a Japanese isolate of ASGV (Yoshikawa *et al.*, 1992) and the related citrus tatter leaf virus (Yoshikawa *et al.*, 1993). The pair that worked best for the detection of UK isolates of ASGV in *C. quinoa* was used for detection in woody hosts. ASGV-A (5' GTT ACT TCC TGG GCA GGT GTT C 3', corresponding to nucleotides 3613-3634) and ASGV-B (5' GCC TCC TTG GGT

CTA TCT TGT A 3', corresponding to the reverse complement of nucleotides 4348-4369) amplify a 757 bp PCR product within the putative viral polymerase gene. PCR microtitre plates were coated with virus-specific $F(ab')_2$ fragments and immunocapture and PCR were performed as for PDV except that conditions for primers A and B were: 42°C for 45 min, 92°C for 2 min followed by 35 cycles of 92°C for 1 min, 56°C 1 min, 72°C 2 min.

All PCR reactions were performed in an OmniGene Temperature Cycler (Hybaid). Eight µl per reaction were analysed in 1.5% agarose gels in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) containing 0.5 µg/ml of ethidium bromide and pGEM size markers (Promega).

PCR product cloning and riboprobe synthesis

PCR products from PDV in cucumber and ACLSV in *C. quinoa* were cloned into the T-tailed EcoRV site of the pMOS*Blue* T plasmid vector (Amersham) according to the manufacturer's instructions.

Sma1-cut and phenol/chloroform extracted DNA from clones pPDVEM1 and pCLSVEM1 served as templates for synthesis of digoxigenin-labeled cRNA probes by *in vitro* transcription using the DIG RNA Labelling Kit SP6/T7 and T7 RNA Polymerase (Boerhinger Mannheim) according to the manufacurer's instructions except that unincorporated DIG-11-UTP was sometimes removed with a commercial spin column (RNeasy, Qiagen).

Dot blot hybridisation

Aliquots of 100 µl per sample extract were applied in duplicate to GeneScreen Plus nylon filters (Du Pont) and baked for 20 min at 120°C and either stored at 4°C for up to one month or processed immediately in the roller tubes of a hybridisation oven (OV 1, Biometra). Filters were pre-hybridised for 1 h at 75°C in 15 ml of 5X SSC (0.75 M NaCl, 75 mM sodium citrate pH 7.0) 1.0% SDS. The solution was replaced with 5 ml of fresh pre-hybridisation buffer containing 50-100 ng/ml of digoxigenin-labelled cRNA probe and hybridised at 75°C overnight. Filters were washed twice for 30 min at 68°C with 50-100 ml of 0.5X SSC, 0.1% SDS. The labelled cRNA probes were detected by enzyme-linked immunoassay with anti-digoxigenin alkaline phosphatase conjugate and subsequent enzyme-catalysed chemiluminescence using Lumigen PPD as substrate (DIG Nucleic Acid Detection Kit, Boehringer Manheim). Membranes were incubated at 37°C for 20 min and exposed to X-Ray film for 2-4 h.

Sequencing

Clone pPDVEM1 was sequenced on an ABI 373A DNA Sequencer (University of Durham, Department of Biological Sciences).

RESULTS

Apple chlorotic leaf spot *Trichovirus* (ACLSV)

Shoots were taken from trees during the winter and forced into growth in the laboratory after a period of winter dormancy; samples of young leaves were tested by IC-PCR and by ELISA. A total of 61 trees were sampled and ACLSV was detected by PCR in one quince, eight apples and eleven plums compared to one, six and seven respectively by ELISA. This confirmed that the primers react with a broad spectrum of ACLSV isolates.

Tests conducted on extracts taken from two apple, one plum and one quince throughout the growing season also showed the superiority of IC-PCR compared to ELISA. In May, samples were taken from the laminae of young and old leaves and from their petioles. Best results were obtained for samples from leaf laminae and these were the only tissues sampled in subsequent months. Each month, ACLSV was detected in more samples by IC-PCR than by ELISA and detection by ELISA was negligible in August and September while 70% of samples were positive in PCR assays (Table 1). Most of the failures to detect ACLSV by IC-PCR were in quince samples. Although all assays were positive in May there was less amplification product in the samples from quince, compared to apple and plum, and the product could often be seen after electrophoresis in only one of the duplicate assays. There was no detection of ACLSV in any samples from quince in August and September by either assay.

A digoxigenin-labeled cRNA probe derived from the PCR product from ACLSV-infected Myrobalan plum was used in hybridisation assays. These detected the virus in extracts from *Chenopodium quinoa* at dilutions of 1:5000 but not in extracts from trees.

Table 1. Monthly tests for ACLSV in field-grown trees by two assays. Forty leaf extracts (ten from each of two apple trees a plum and a quince) were tested each month.

Month	Percent samples positive by:	
	ELISA	IC-PCR
May	70	100
June	90	98
July	25	75
August	5	70
September	10	70

Apple stem grooving *Capillovirus* (ASGV)

The detection of ASGV in field samples of apple and oriental pear was poor by IC-PCR and only 47% of samples were positive in May, compared to 70% by ELISA. The PCR reactions were weak in most cases and as there were no positives in any of the June samples, further PCR assays were not attempted on samples from fruit trees. In contrast, 82% of the samples were positive by ELISA in June and 53% in August.

Prune dwarf *Ilarvirus* (PDV)

All three assays detected PDV successfully in virtually all samples collected from cherry and plum trees in the early part of the growing season (Table 2). However, from July onwards detection of PDV became difficult by ELISA in extracts from cherry and it was not detected in any cherry samples in August and September although it was easily detected in most of the samples from plum. The results of assays of plum extracts by IC-PCR and hybridisation were similar to ELISA except that both PCR and hybridisation detected PDV in most of the cherry samples in August and over half of them in September (PCR: 11 of 20, hybridisation: 13 of 20 samples); samples from young and old leaves gave similar results.

The PCR product amplified from isolate 4/4 had 95% sequence in common with this region of the coat protein gene of the US isolate of PDV described by Bachman *et al* (1994).

Table 2. Monthly tests for PDV in field-grown trees by three assays. Forty leaf extracts (ten from each of 2 cherry and 2 plum trees) were tested each month.

Month	Percent samples positive by:		
	ELISA	IC-PCR	Hybridisation
May	100	100	100
June	95	100	100
July	65	100	100
August	48	93	93
September	50	78	78

DISCUSSION

The requirements of tests applied for the Certification of fruit trees are particularly stringent. Infection is often latent and vegetative propagation from, for example, an infected apple scionwood mother tree (providing material to propagate several hundred trees per year) can result in widespread infection that is difficult to locate if detected several years after it has arisen. Tests are needed that detect a wide range of virus strains with maximum reliability.

Our results show that IC-PCR is reliable over a large part of the growing season for the detection of ACLSV. It is also effective with a wide range of ACLSV isolates despite the variability of the virus genome. These conclusions are in agreement with the extensive studies of Candresse *et al.* (1995) who designed the primers and used a similar protocol to detect ACLSV in an average of 79% of samples taken from orchard trees of apple, pear, plum, cherry, apricot and peach in southern France from April to November. They had a low detection rate in pear as we did in the closely related quince; this may be due to a low concentration of virus as poor results were obtained with ELISA as well as by PCR.

The poor performance of our PCR assay for ASGV in fruit trees was unexpected. Preliminary results (to be published elsewhere) of sequencing the 757 bp PCR products from the putative polymerase gene of our two ASGV isolates (SG and Wijcik), amplified from infected *C. quinoa* by the primers used in the field study, indicate that improved primers may give better results. The two isolates from the UK had over 95% of sequence in common but they both had only about 80% in common with the Japanese isolates of ASGV and citrus tatter leaf virus that were used to design the primers.

In common with others (Scott *et al.*, 1992; Torrance & Dolby, 1984) we found serology to be reliable for the detection of PDV early in the growing season but not later; both hybridisation and PCR were effective until late summer. The IC-PCR protocol is relatively simple in comparison to the RT-PCR procedure of Parakh *et al.* (1995) as plant samples are extracted in the same way as for ELISA and immuno-capture, reverse transcription and amplification are all conducted in a single PCR microtitre plate. For reasons that are unclear, poor results were obtained if this procedure was used for ACLSV and for this virus IC-PCR was satisfactory in our hands only when conducted in microfuge tubes. The dot blot hybridisation protocol for PDV was also convenient; crude plant extracts were applied to the membrane, simple buffers without formamide were used, following the method of Barbara *et al.* (1990) for the detection of hop latent viroid and the probe was non-isotopic. The success of this protocol for PDV and not for ACLSV is probably due in part to the degree of conservation between isolates of the respective viruses in the regions comlementary to the probes.

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DIFFERENTIATION OF PHYTOPLASMAS ASSOCIATED WITH SWEET POTATO LITTLE LEAF DISEASE AND OTHER PHYTOPLASMAS IN THE FABA BEAN PHYLLODY CLUSTER

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ABSTRACT

Isolates of sweet potato little leaf phytoplasma (SPLL) were obtained from countries in S.E.Asia and the western Pacific and established in a glasshouse at HRI-East Malling. Antisera were raised and used to characterise the isolates and to investigate their serological relationships with other phytoplasmas. PCR and RFLP analyses were carried out. No serological differences were observed among SPLL isolates but all were different from peanut witches' broom, tomato bigbud, faba bean phyllody and other phytoplasmas in this group, both by ELISA and, for those examined, by Western blot profiles. A plate capture-PCR detection method was able to detect SPLL in dilutions of sweet potato extracts greater than 1:10,000. The relevance of these results to SPLL epidemiology is discussed.

INTRODUCTION

Sweet potato *(Ipomoea batatas)* is a staple food crop in many countries in S.E.Asia and the Pacific region. Severe crop reduction can be caused by the disease 'Little Leaf' (SPLL), sometimes called 'Witches' Broom', a phytoplasma infection first described from the Ryukyu Islands of Japan (Summers, 1951). The disease names are descriptive as affected plants are very weak, have shortened stolons and proliferated axillary growth, and the leaves are very small in comparison with unaffected plants. Tubers are small and thin and in severe infections may not grow at all. Serological relationships have been reported with phytoplasmas associated with peanut and asparagus bean witches' broom diseases as well as with several weed species (Shen & Lin, 1993). Comparisons of RFLP profiles of PCR-amplified 16S-rDNA products from SPLL and tomato bigbud (TBB) phytoplasmas in Australia indicated a close relationship (Gibb *et al*, 1995). As part of a programme to improve the health of planting stock a project was undertaken by HRI, East Malling, funded by ODA-NRI, to characterise and compare the phytoplasma isolates from sweet potato crops and to determine their relationship with other phytoplasmas. The results obtained would be used to select and develop appropriate diagnostic reagents and procedures to detect and identify the phytoplasmas in plant hosts and insect vectors.

MATERIALS AND METHODS

Plants and phytoplasma isolates

Isolates of SPLL phytoplasma in sweet potato were obtained from Indonesia, Papua New Guinea, New Caledonia, Vanuatu and Australia during 1994 and 1995. More than 20 different isolates were successfully established in a quarantine glasshouse at HRI-East Malling, the majority of which were then graft-transferred to plants of a clonal sweet potato selection, kindly supplied by Dr Sinclair Mantell of Wye College, University of London. Most field-collected source plants were also infected with one or more viruses, but these appeared not to interfere with the subsequent characterisation of the various phytoplasma isolates. Attempts at HRI-East Malling to transfer SPLL phytoplasmas into *Catharanthus roseus* L. or tomato by interspecific grafts or with various *Cuscuta* species were unsuccessful, although an infected *C.roseus* plant obtained from Australia was initially thought to have been infected via *Cuscuta australis* transfer from an SPLL-infected sweet potato plant (isolate KGCr); consequently, sweet potato was used as the source of all SPLL phytoplasma extracts and preparations. Other phytoplasmas involved in the study (Table 1) were grown and passaged in *C.roseus*, except for TBB which was also grown in tomato. Plants were kept in a quarantine glasshouse at East Malling maintained at 25-28°C with supplementary lighting.

SPLL Purification and Antibody Production

An SPLL isolate obtained from New Caledonia (NC1) was selected for detailed study, on the basis of symptom severity and the concentration of phytoplasmas observed by UV microscopy of DAPI-stained cryo-sections. Several protocols were utilised for the partial purification of phytoplasmas from plant extracts. Rabbits were immunised with each preparation, emulsified either with Freund's incomplete adjuvant (FIA), Imject Alum (Pierce Chemical Co..) or Hunter's Titermax (CytRx Corporation). Highest titres, measured by indirect ELISA (see below), were obtained following a relatively simple differential centrifugation protocol (Sarindu & Clark, 1993) and immunisation with FIA. Antisera with suitable activity were cross-absorbed with equivalent healthy sweet potato plant extracts and IgG prepared immediately by ammonium sulphate precipitation and filtration through a column of DE52 cellulose (Whatman Biochemicals) (Clark & Adams, 1977). Indirect Elisa tests were made using the $F(ab')_2$ procedure described by Barbara & Clark (1982). Following addition of the protein A-horseradish conjugate (PA-HRP) and tetra methyl benzidine substrate (TMB), colour development was stopped by addition of 3N H_2SO4 and reactions were measured using a model 3550 plate reader (BioRad Ltd).

Phytoplasma membrane protein preparations were separated by SDS-PAGE in 12% discontinuous gels in a mini-Protean II gel apparatus (BioRad Ltd) using standard Laemmli buffer and electrophoresis conditions. The separated proteins were electroblotted to 0.2 or 0.45 µm nitrocellulose sheets and probed with cross-absorbed IgG preparations from selected phytoplasma antisera. Blots were developed with protein A or goat anti-rabbit antibodies conjugate with alkaline phosphatase (ALP) and developed, following blocking and extensive washing , with naphthyl-1-phosphate and Fast Red TR substrate.

DNA preparation and analysis

DNA was prepared for PCR amplification by the method described by Ahrens and Seemuller

(1992). PCR investigations were made either with 'universal' 16S-rDNA primers U5/U3 (sequence information kindly supplied by Dr Erich Seemuller), or with sweet potato witches' broom-derived primers (SP3A/SP4B) synthesised from sequences published by Ko and Lin (1994). DNA was subjected to PCR amplification for 30 cycles in reaction volumes of 20 µl in thermostable microtitre plates (Hybaid Ltd). Conditions for use with the U5/U3 primers were 95°C for 0.5 min, 50°C for 0.5 min and 72°C for 0.5 min with a final extension step of 72°C for 2 min. These primers gave a product of about 880 bp. For amplification with the SP3A/SP4B primers the corresponding conditions were 94°C for 25 sec, 46°C for 20 sec and 72°C for 25 sec. These primers amplified a fragment of about 1.1 kbp. For all PCR amplifications a $MgCl_2$ concentration of 1.5 mM was used with 0.2 mM of each dNTP and 0.5 units of Taq polymerase. RFLP analyses were carried out on U5/U3-amplified PCR products following digestion with various endonucleases.

RESULTS

Serological characteristics and relationships with other phytoplasmas

All SPLL isolates gave positive ELISA reactions with the polyclonal antisera raised against isolate NC1, but considerable variation in the strength of the ELISA values was observed from test to test. This may have resulted from possible interference with the reactions by latex-like components in the plant extract. Negative reactions were recorded with healthy sweet potato extracts. ELISA tests were carried out with other phytoplasmas and IgG preparations from homologous polyclonal antisera (Table 1). No positive reactions were obtained in reciprocal tests between SPLL antibodies or preparations with any of the other phytoplasmas examined, with the possible exception of a very weak cross reaction with TBB. The TBB reaction was recorded only in some ELISA tests and was usually accompanied by a high background reaction against healthy plant extracts, making interpretation difficult. No reactions were recorded between SPLL antibodies and extracts of peanut witches' broom plants which were DAPI positive and which showed strong proliferation symptoms. However, as no PWB antisera was available these apparent negative reactions could not be confirmed.

Western blot analyses of affinity-purified SPLL and other phytoplasma membrane proteins revealed the presence of a single major protein for each of the phytoplasmas (Fig.1). SPLL isolates were distinguished from all the other phytoplasmas studied, including TBB, both by the specificity of the SPLL antibody reaction and by differences in the observed molecular size of the respective immuno-stained protein. The major protein band from SPLL isolates showed an approximate molecular size around 39 kDa, whereas the molecular size of the TBB protein was around 19 kDa and those of FBP, BLTVA, SP and CrWB were all about 20 kDa.

PCR and RFLP analyses

Products were obtained from all SPLL isolates and other phytoplasmas in PCR reactions using the U5/U3 'universal' primers. Using SP3A/SP4B primers from Taiwan, SPLL isolates could be assigned to one of two broad groupings, according to their geographic origin; those from New Caledonia and Vanuatu amplified well and were placed in one group, while those from Australia, Indonesia and Papua New Guinea did not amplify and were placed together

in a different group (Table 1). Interestingly, these primers amplified DNA fragments also from *C.roseus* extracts of TBB, CrWB, SesP, JutP, FBP and BLTVA but not of CrP or SoyP.

RFLP analysis of U5/U3-primed products showed all isolates to be closely related to TBB from Australia (Table 1) and to other members of the faba bean phyllody cluster, but precise relationships varied according to the restriction enzyme employed. Preliminary Southern blot analyses of EcoR1 digests with a cloned fragment from TBB DNA indicate the possibility for further differentiation of isolates (results not shown).

Immuno-capture and plate-capture PCR detection

Quantitative inconsistencies in the results of ELISA tests with SPLL isolates prompted an investigation of the use of immuno-capture PCR (IC-PCR) as a possibly more reliable and sensitive detection method. A series of tests were carried out using PCR tubes and thermostable 96-well plates with homologous and heterologous combinations of various phytoplasmas and antibody preparations. Immunocapture was followed by PCR amplification using either U5/U3 primers or SP3A/SP4B primers, following the method described by Rajan & Clark (1994). Amplification of all phytoplasmas was obtained with U5/U3 regardless of the antibody used to capture the phytoplasma. Equally sensitive detection was obtained without the use of a capture antibody. This result is probably a consequence of the ineffectiveness of the washing procedure in the necessary absence of Tween 20 or other detergent following 'immuno-capture'. Post-coat 'blocking' of attachment sites with various blocking agents did not effectively control non-specific attachment of phytoplasma. Some degree of specificity for the target phytoplasmas could be achieved using specific primers. Detection of SPLL phytoplasma DNA was obtained at dilutions of plant extract, in phosphate or glycine buffers, greater than 1:10,000.

DISCUSSION

Many thousands of cultivars and local selection of sweet potato are grown in countries in S.E.Asia and the western Pacific region. Little leaf disease can be a major cause of significant yield loss. As with all vegetatively propagated crop plants the health of the planting stock dictates the primary disease profile of the newly-planted crop while epidemiological aspects of pathogen dissemination within and between crop and weed hosts need to be known to prevent further spread. The results of this study reveal some diversity among the isolates obtained, with an indication of a geographic division into two groups. In one or more analytical respects all the SPLL isolates examined appear distinct from other phytoplasmas in the study including, probably, several isolates of peanut witches' broom, reported elsewhere to be serologically related to SPLL. PCR and RFLP investigations in this study and reported by Padovan *et al* (1994) failed to differentiate SPLL from TBB phytoplasmas, but the heterologous reactions observed in ELISA tests, together with the different Western blot profiles show these phytoplasmas cannot be considered the same organism. For this reason an ELISA-based procedure would be preferred as a detection and identification method for field infections but so far this technique has not proved sufficiently reliable with sweet potato extracts to guarantee accurate results. Improvements in the quality of the antibodies used could help overcome this problem. In the meantime rapid and sensitive detection is possible by a simple plate capture PCR method, in which specificity of

detection is dependent upon the selection of appropriate primers.

Table 1. Numbers and source of SPLL phytoplasma isolates, and serological relationships, PCR characteristics and RFLP profiles in comparison with other related phytoplasmas.

PLANT SOURCE	PHYTOPLASMA ISOLATE/ORIGIN	U5/U3 PCR - RFLP		SP3A/4B PCR	SEROTYPE
		TaqI	Tru9I		
Sweet Potato	New Caledonia (3)	A	C	+	SPL
	Vanuatu (10)	A	C	+	SPL
	Papua New Guinea (4)	A	C	-	SPL
	Indonesia (3)	A	C	-	SPL
	Australia (1)	A	C	-	SPL
C.roseus	Tomato Bigbud (TBB)	A	C	+	TBB
	Crotalaria Witches' Broom (CrWB)	A	C	+	CrWB
	Sesame Phyllody (SesP)	B	C	+	CrWB
	Cleome Phyllody (CmP)	B	C	+	CrWB
	Jute Phyllody (JuP)	B	C	+	CrWB
	Crotalaria Phyllody (CrP)	A	D	-	FBP
	Soyabean Phyllody (SoyP)	A	D	-	FBP
	Faba Bean Phyllody (FBP)	A	D	+	FBP
	BLTVA*	B	C	+	CrWB
	VCPT**	A	D	-	FBP

* Beet Leafhopper Transmitted Virescence Agent
** Periwinkle bait plant from Tanzania

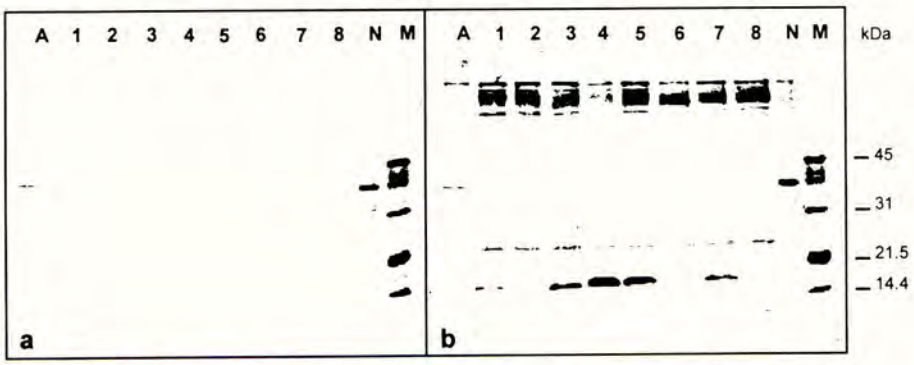

Fig. 1. Western blot profiles of partially purified SPLL and other phytoplasma membrane proteins: **a**, probed with SPLL IgG; **b**, probed with a mixture of SPLL, TBB, CrWB and FBP IgGs. A=Australian SPLL, N=affinity purified NC1 from sweet potato; 1=KGCr, 2=TBB from Australia, 3=TBB from East Malling, 4=BLTVA, 5=SesP, 6=FBP, 7=CrWB, 8=healthy *C.roseus*; M=MW markers. Nos 1-8 were preparations from *C.roseus*.

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DETECTION OF PHYTOPLASMAS ASSOCIATED WITH PEAR DECLINE IN PEAR PSYLLIDS BY POLYMERASE CHAIN REACTION

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ABSTRACT

Polymerase chain reaction was used to detect phytoplasmas in pear psyllids (*Cacopsylla pyricola)*, the insect vector of pear decline, using primers to sequences in the 16S and 23S RNA gene. Psyllid samples from eight commercial pear orchards of the cv. Conference and from one experimental plot of pear seedlings were tested over a period of one year. There was no correlation between either the numbers of pear psyllids or the proportion of pear psyllids yielding a positive result by PCR and the level of pear decline in the commercial orchards. The proportion of psyllids carrying phytoplasmas varied from less than 1% to approximately 40% depending upon the source and the time of year. The highest proportion of insects indexing positive occurred in late summer when the concentration of phytoplasmas in pear trees was also high. However, positive results were also obtained from overwintering adults sampled in spring, a time of the year when phytoplasmas cannot be detected in the aerial parts of pear trees. A model explaining the spread of phytoplasmas associated with pear decline is proposed.

INTRODUCTION

Pear decline (PD) is widespread throughout N. America and Europe and is a major problem in the UK in young pear (*Pyrus communis)* orchards of the cv. Conference with quince rootstocks. The disease is associated with phytoplasmas (the recently adopted trivial name for mycoplasma-like organisms) and is spread by the pear psyllid *Cacopsylla pyricola*, the most common insect pest in pear orchards.

Phytoplasmas in pear trees require a fully functioning phloem to survive and multiply (Schaper and Seemuller, 1982) and are usually only readily detected in the late summer and autumn when the premature reddening symptoms of the disease are apparent. The phytoplasmas die away from the aerial parts of pear trees during the winter and are completely absent in spring when the more damaging symptoms of pear decline can be seen. They can survive in the roots of pear seedlings or pear rootstocks during the winter from where they recolonise the rest of the tree each growing season. However the majority of commercial pears in the UK are grown on quince (*Cydonia oblongata)* rootstocks, a host not readily colonised by phytoplasmas. On this rootstock a partial or complete loss of phytoplasmas takes place each winter.

The epidemiology of the disease is poorly understood: Trees protected completely from pear psyllids remain phytoplasma-free but complete control of pear psyllids under

commercial conditions is impossible. The psyllid life cycle comprises two summer generations and one winter generation per year, and each generation consists of 5 larval stages and a mobile adult stage. The psyllids survive the winter as adults. The level of psyllid control to minimise feeding damage is well understood but the level of control to limit the spread of PD is unknown. There is no clear correlation between psyllid numbers and the level of PD, both of which can vary considerably from site to site. Trees protected from early season populations of pear psyllids may become infected later in the year but remain free of the spring symptoms of PD the following year (Davies *et al,* 1992) implying that overwintering pear psyllid adults play an important role in the spread of the disease.

Most work with PD has relied upon UV light microscopy for the detection of phytoplasmas, but this technique is unable to distinguish between different phytoplasmas and is unsuitable for use with pear psyllids. This has placed a major constraint on progress to study the epidemiology of PD. However, PCR techniques are now available for the detection of phytoplasmas, either by using primers to sequences in the highly conserved 16S RNA gene, or by using primers based on sequence data from oligonucleotide probes. Primers are available for the detection of phytoplasmas associated with pear decline by PCR (Lorenz *et al*, 1995) that can also be used for the detection of phytoplasmas in pear psyllids (Davies *et al,* 1995). The aim of this work was to utilise PCR to determine the proportion of psyllids from a range of orchards that were carrying phytoplasmas in comparison with psyllid numbers and pear decline levels, and to search for a pattern that could be exploited in developing a control strategy.

MATERIALS AND METHODS

Psyllid collection

Eight orchards were selected, containing trees between three and seven years old, of the variety Conference with quince rootstocks. A block of c.1000 trees was chosen from each site for the collection of psyllids and the recording of PD symptoms. Psyllids were also collected from an experimental plot of pear seedlings where a high level of phytoplasma infection was known to be present. Psyllids were collected by limb jarring: a sharp tap was applied to one limb of each pear tree and falling insects were collected in a 1m^2 nylon funnel. Pear psyllids were separated from other fauna using an aspirator. Psyllids were collected from between 100 and 200 trees from each site.

DNA preparation

DNA was prepared from pear psyllids, either singly or in groups of five, by a modification of the technique described by Edwards *et al* (1991).

Primers

The primers used are listed in Table 1. P1/P7 amplify a 1800bp sequence from a wide range of phytoplasmas. f01/r01 amplify a 1000bp sequence from most European fruit tree phytoplasmas. fPD/rPD amplify a 1400bp sequence from PD phytoplasmas only.

Table 1. Primers

Primer	Target	Position	Reference
P1	16SrDNA	7 - 29	Deng and Hiruki, 1991
rP7	23SrDNA	1818 - 1836	Kirkpatrick(unpublished)
f01	16SrDNA	65 - 91	Lorenz *et al*, 1995
r01	16SrDNA	1115 - 1136	Lorenz *et al*, 1995
fPD	16SrDNA	204 - 224	Lorenz *et al*, 1995
rPD	16S/23S spacer	1600 - 1620	Lorenz *et al*, 1995

Polymerase chain reaction

DNA (1-5ng) was subjected to PCR for 35-50 cycles in reaction volumes of 20μl. The PCR conditions were 93^{0}C for 1 minute, 52^{0}C for 1 minute and 72^{0}C for 1 minute with a final extension step of 72^{0}C for 10 minutes. A $MgCl_2$ concentration of 1.5mM was used with 0.2mM of each dNTP and 0.5 units of Taq polymerase. When performing nested PCR a preliminary stage 15 or 20 cycles was carried out using primers P1/P7 and 0.5μl of the product was transferred to fresh reaction mixture. Amplification products were analysed by electrophoresis on 1.5% agarose gels containing 0.5μg/ml ethidium bromide and viewed with a UV transilluminator.

RESULTS

The proportion of trees with spring symptoms of PD at the eight orchards examined varied from 3% in the least affected orchard to 16% in the most severely affected (Table 2). The number of trees with autumn symptoms recorded later the same year varied from 3% to 27%. The numbers of pear psyllids occurring in the eight orchards throughout the year also varied considerably (Table 2) but there was no correlation between the level of psyllid control and the levels of PD.

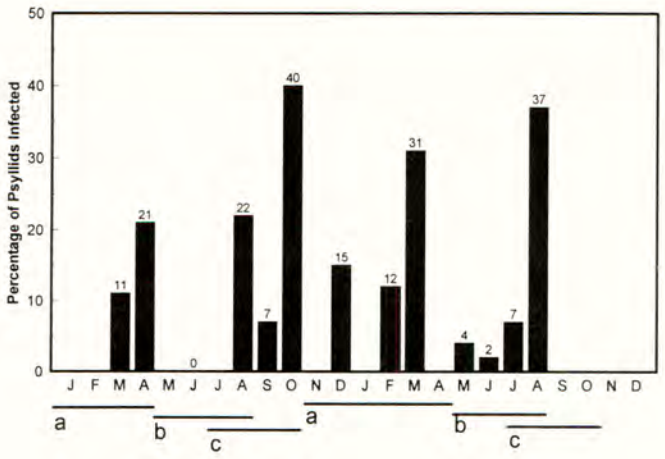
PCR using fPD/rPD or f01/r01 primers yielded similar results with DNA prepared from psyllids collected from an experimental pear seedling plot, a site where a high level of phytoplasma infection was present. None of the insect DNA samples from this site yielded a product with the less specific f01/r01 primers that did not also give a product with the fPD/rPD primers, despite reports (Lorenz *et al,* 1995) that some isolates of PD are not amplified by the PD primers. Nevertheless, it was considered prudent to use the f01/r01 primers for the majority of field testing to reduce the risk of any PD isolates remaining undetected. Early PCR experiments showed that direct PCR was usually sensitive enough to yield a product from single insect DNA preparations. Nested PCR experiments were found to be even more sensitive, occasionally yielding a positive result not seen by direct PCR. Most experiments were performed with DNA extracts made from adult psyllids, the stage considered most likely to be involved in the spread of phytoplasmas. However, DNA made from the larval stages that had fed on infected plants also yielded a product by PCR.

PCR analysis of pear psyllid samples was carried out on DNA prepared from 20 groups of 5 insects collected from the 8 orchards and the pear seedling site at different times of the year. The proportion of psyllids carrying phytoplasmas was estimated by a method of maximum likelihood (Thompson, 1962). The numbers of psyllids carrying phytoplasmas from the 8 commercial orchards was usually low (Table 2) and less than 1% of the insects tested in many cases. Highest levels were recorded in the spring from overwintering adults, and in late summer. There was no clear correlation between the proportion of infected psyllids and the levels of PD at the 8 sites.

Table 2. PD symptoms, pear psyllid numbers, and proportion of phytoplasma+ pear psyllids from 8 commercial pear orchards.

	1	2	3	4	5	6	7	8
Spring symptoms(%)	6.1	5.4	6.6	4.3	4.8	6.1	16.4	3.4
Autumn symptoms(%)	6.1	10.8	19.8	13.5	3.6	9.4	27.7	7.8
Psyllids/10 beats,spring	9.8	5.7	2.0	28.8	4.0	61.0	8.6	16.0
Phyto+ psyllids(%),spring	6.25	<1	5.6	2.1	2.1	4.4	<1	2.1
Psyllids/10 beats,autumn	25.7	40.0	80.0	60.0	40.0	43.3	23.3	76.7
Phyto+ psyllids(%),autumn	9.7	<1	<1	2.1	<1	<1	23	<1

Fig.1. Proportion of pear psyllids carrying phytoplasmas collected from pear seedlings throughout the year. Horizontal bars represent approximate periods of (a) overwintering generation adults,(b) first summer generation adults and (c) late summer generation adults.



The proportion of psyllids that yielded a PCR product was much higher from the pear seedling plot than from the commercial orchards (Fig.1), and reflected the levels of phytoplasmas occurring in the seedlings throughout the year. Approximately 40% of psyllids collected in late summer, when phytoplasma levels are at their highest in the plants, indexed positive. The lowest proportion of psyllids carrying phytoplasmas occurred in late spring/early summer when the first spring generation of adults was feeding. There was also a high proportion (c.20%) of overwintering adult psyllids collected in spring that yielded a product, a time of the year when phytoplasmas are completely absent from the aerial parts of pear trees.

DISCUSSION

A comparison of PD symptoms and pear psyllid levels at the 8 pear orchards confirms previous observations (Davies and Guise, unpublished) that there is no direct correlation between overall psyllid numbers and the level of pear decline. There is also no apparent correlation between the proportion of psyllids carrying phytoplasmas detected by PCR and the levels of pear decline, although the numbers of psyllids indexing positive from the 8 orchards were very low compared with psyllids collected from the pear seedling plot. The low readings reflect the low levels of phytoplasma infection found in pear orchards where the trees are grown on quince rootstocks. The seasonal disappearance of phytoplasmas from such trees results in only about 3-30% of trees being infected in any one year, whereas the survival of phytoplasmas in the roots of pear seedlings eventually results in all trees becoming infected. The concentration of phytoplasmas in pear seedlings is also higher than that occurring in trees with quince rootstocks.

Clearer results were obtained from psyllids collected from the pear seedling plot where the build up of phytoplasmas in the aerial parts of the pear seedlings throughout the summer was reflected in an increased proportion of psyllids yielding a positive result by PCR. However, at most of the sites examined, some of the overwintering psyllids collected in spring yielded a product by PCR at a time when phytoplasmas are absent from the aerial parts of pear trees, suggesting that the psyllids had either acquired the phytoplasmas by feeding on infected pears the previous autumn, or had acquired the phytoplasmas from a different source during the winter. There is evidence that overwintering pear psyllids can migrate and survive on other woody hosts (Hodgson and Mustafa, 1984), but there is no evidence that they can feed on other hosts and there is no record to date of the phytoplasmas associated with pear decline occurring in any other host. It is likely that overwintering adults play an important role in the disease cycle by retaining phytoplasmas that are otherwise lost from pear trees during the winter months. This is consistent with the finding that pear trees protected from early season populations of pear psyllids remain free of pear decline the following year (Davies *et al*, 1992).

There is insufficient evidence to suggest a level of psyllid control that would also effectively limit the spread of phytoplasmas in pear orchards. An obvious control target would be the overwintering generation insects but it is also possible that summer generations play an important role by spreading phytoplasmas within infected trees and from infected trees to uninfected trees. Given the evidence accumulated to date the following model for the spread of phytoplasmas in pear trees may be proposed.

Autumn: The first autumn reddening symptoms appear in phytoplasma infected trees. Overwintering generation larvae and adult psyllids acquire phytoplasmas by feeding.

Winter: Phytoplasmas die away from trees during dormancy. Winter generation adult psyllids survive and retain phytoplasmas until spring.

Spring: Pear trees infected the previous autumn display spring symptoms of pear decline. Phytoplasmas absent from pear trees, but present in surviving psyllids and reintroduced into pear trees by feeding.

Summer: Phytoplasmas multiply in pear trees. First and second generation psyllid larvae and adults feed and further spread phytoplasmas within trees and to new trees.

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