Project title:	The incidence and etiology of pear stony pit disease
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Key words:	Pear, stony pit, Apple stem pitting virus, ASPV, PCR, diagnostics

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Grower Summary

Headline

- A grower survey indicated that pear loss from stony pit disease in orchards may amount to almost £300k per annum. However various factors mean that the significance of the problem may be understated.
- Improvements in virus detection have not reached the stage at which a rapid diagnostic approach can be offered to the industry. Therefore there is no immediate financial benefit or any new actions to be taken.

Background and objectives

Fruit affected by stony pit are worthless with pits on the surface and hard, stony lumps in the flesh. The fruit can be severely misshapen. Symptoms vary in severity between seasons and from a few to most of the fruit on a tree may be affected. In recent years samples were received from several orchards in East Anglia and Kent. These were from young trees (less than 7 years old) as well as from old ones of uncertain health status when planted. In one orchard there appeared to have been extensive spread into trees about 5 years old. The symptoms were seen on cultivars Beurre Hardy, Conference, Doyenne du Comice and Concorde. They are often attributed to boron deficiency and diagnosis can be uncertain without a confirmatory graft test. This is usually impractical as it takes at least 3 years to complete. It was not known how common the disease is and it was proposed to gather information on its occurrence in commercial orchards.

The disease is caused by a virus or virus-like agent and has been associated with *Apple stem pitting virus* (ASPV) by some workers. This virus is widespread in old trees not originating from virus-tested material and the association may therefore be spurious.

The objectives of the project were to:

1. Determine the incidence and economic importance of Pear Stony Pit disease in commercial pear orchards in the UK

2. Gather information on factors affecting the incidence of Pear Stony Pit disease in commercial orchards

3. Establish whether there is a strong correlation between Pear Stony Pit disease and Apple Stem Pitting Virus (ASPV)

4. Provide guidance on disease prevention and control from the best available information

Summary of the project and main conclusions

RT PCR primer design

The project aimed to provide a molecular diagnostic test for ASPV. A PCR-based test for ASPV detection was developed and validated (see the Science Section of the report). The probes specific to ASPV were designed to ensure that the test is highly specific for this virus and adaptable to detect a range of strains, with a possibility to detect previously uncharacterised ASPV strains.

ASPV detection test validation

To assess sensitivity and specificity of the test procedure, ASPV was tested in two pear trees showing severe stony pit symptoms (for which ASPV presence was previously confirmed) and in two healthy pear trees from the HRI collection. One combined sample per tree included bark from young branches, fruit epidermis, and leaf material. Isolation of the RNA from the sampled material was carried out using a commercially available RNA isolation kit as described in the science section. Reverse transcription reaction and PCR reactions were carried out as described in the science section. The specific result expected was detected only in the case of samples isolated from pear trees showing stony pit symptoms (2 out of 2 trees). No positives were detected in the case of samples isolated from the apparently healthy pear trees. Those results provided initial indications

that pear trees with stony pit symptoms from the HRI collection contained an ASPV strain closely related to those already known. The results also provided initial confidence in the viability of the detection procedure.

Whilst validating the test, material collected from reference trees was specifically kept for several hours at room temperature prior to RNA isolation to mimic the time delay to which samples could be subjected when sent by post.

Test of samples provided by growers for the presence of ASPV

By using the test procedure outlined in this report, two pear samples with suspected mild stony pit disease submitted by a grower were tested. Both samples proved ASPV positive, although levels of ASPV were lower than in reference material showing severe symptoms. In the control samples isolated from healthy pears, no ASPV was detected.

Although there was a definite link between the presence of ASPV in pear exhibiting stony pit symptoms, it was not known how common the disease is. To gather information on its occurrence in commercial orchards a survey was prepared and distributed to growers.

Grower survey to determine the incidence and severity of pear stony pit disease in UK orchards

The response rate to the survey was 59.8%. Of those who responded to this survey 57% reported some level of infection with stony pit disease in their pear orchards with 69.5% of the reported orchard area being affected. However, only 4.7% reported greater than 6% of trees affected, representing some 2.15% of the area reported. Incidence was geographically equivalent throughout UK production areas although Sussex and the West Midlands (Herefordshire; Worcestershire, Gloucestershire) appeared to have a higher proportion of affected trees. Factors highlighted by the survey included considerable variability from season to season, infection in both old and young trees and similarity in symptom expression between stony pit disease and boron deficiency.

Extensive sampling of leaf, bark, stem, and fruit was made from six sites, culminating in 140 samples to test by PCR. Ninety-nine of these samples were from one site with a history of symptoms. Initial results indicated that the probes were too specific, i.e. they missed some virus strains in known positive samples. Therefore new probes were designed and tested.

Results showed that:-
Probes and symptoms match (+ and + or - and -) on 45% of occasions.
Probes detect in plants without symptoms on 35% of occasions.
On 20% of occasions, there are symptoms, but the PCR was negative.

The results were approximately consistent from site to site. At one site where orchards of varying age were tested, there appeared to be greater incidence of the virus in younger trees (9 years old). This was not apparent as symptoms (which were less than in older orchards), but was detected by PCR. However, the sample size was not large enough to draw too many conclusions from this. This type of data may have relevance to disease spread, and symptom expression when related to the development and productive life of orchards.

This suggests that the current molecular system will detect 4 in 5 cases of pear stony pit disease, although this figure may be lower since one cannot verify results that are negative in symptom and PCR test.

There are many reasons why these figures may be as they are, including the possibilities that symptoms may not have been caused by a virus, the sampling approach has missed the virus, the probes are still missing some strains, or the test needs modification to pick up low levels of virus.

Conclusions

PCR and sequence data from this project provide substantive evidence, but not firm proof, that ASPV is responsible for stony pit disease of pear. However, the variable nature of the virus has meant that development of a reliable molecular test for the disease has not proceeded to the stage at which a commercial diagnostic service can be offered.

Future work should take into account:-

- A nested approach to the PCR with redesigned primers to address the possibility of low virus titre leading to false negatives.
- Several samples should be tested from infected plants (at least three) to account for uneven distribution of the virus.
- By combining the original and redesigned primers it should be possible to ascertain whether sequence variability is causing difficulties with detection, and it may be possible to combine primer sets to counter this potential problem.
- The PCR conditions may be refined to improve detection.

Financial benefits

There are no direct financial benefits to growers as a result of this research. The PCR test has potential to provide a rapid screening approach for production of healthy planting material, but needs further development.

Action points for growers

- There are no new actions for growers as a result of this project.
- However, the surveys performed indicate that stony pit virus is a disease problem in the UK, and hence appropriate testing of propagation material is still important to avoid vegetative transmission.

Science section

Introduction

Fruit affected by stony pit are worthless with pits on the surface and hard, stony lumps in the flesh. The fruit can be severely misshapen. Symptoms vary in severity between seasons, between trees in an orchard, and even from fruit to fruit on an individual tree. From very few to most of the fruit on a tree may be affected. The symptoms have been seen on cultivars Beurre Hardy, Conference, Doyenne du Comice and Concorde, with most widely grown varieties susceptible. However, symptoms may be confused with boron deficiency or mechanical and environmental damage, and diagnosis can be uncertain without a confirmatory graft test. This is usually impractical as it takes at least 3 years to complete.

In recent years samples have been received from several orchards in East Anglia and Kent. These have been from young trees (less than 7 years old) as well as from old ones of uncertain health status when planted. In one orchard there appeared to have been extensive spread into trees about 5 years old. Currently, the only known means of spread of the virus is through contaminated propagation material, and control is by maintaining virus-free sources.

The disease is caused by a virus or virus-like agent and has been associated with apple stem pitting virus (ASPV), initially by graft tests between apple and pear. More recently, serological cross-matching has shown similarity between the causal agents of these diseases (Jelkmann *et al*., 1992; Paunovic *et al*., 1999). The 9.3kb cDNA sequence of the positive sense RNA virus, ASPV, was obtained in 1994 (Jelkmann, 1994). Since then, studies on double-stranded RNA have indicated a close relationship between ASPV and both pear stony pit and quince sooty ringspot virus (the latter apparently identical) (Paunovic *et al*., 1999). However, differences between ASPV and pear stony pit were observed, and it has also been found that within ASPV there is significant genome variability (Schwarz and Jelkmann, 1998). Therefore,

although ASPV and pear stony pit may be one and the same, there may still be considerable differences between strains.

In a recent HortLINK project(HDC project HNS/SF 87) we isolated a strain of ASPV from a tree with stony pit and found that it reacted differently from other sources of the virus in PCR assays. This may therefore be a variant of ASPV that is peculiar to stony pit disease.

Objectives:

1. To gather information on the incidence of the disease in commercial orchards.
2. To establish whether there is a correlation between pear stony pit disease and apple stem pitting virus.

Materials and Methods

Collection of samples for PCR primer design and testing

Two pear trees showing severe stony pit symptoms (for which ASPV presence was previously confirmed) and two healthy pear trees were from sampled from the HRI collection. One combined sample per tree included bark from young branches, fruit epidermis, and leaf material.

Design of PCR primers

Primers for ASPV detection were designed by analysing the coat protein gene sequences of four ASPV isolates available in June 2002. These are shown in Figure 1.

The accession numbers of the four sequences are indicated on the left of Figure 1. The primers were designed to regions containing good homology between isolates, and which were also suitable for producing a reasonable size fragment (250-300 bases) using a low-stringency PCR procedure.

Extraction of viral RNA

The total RNA was extracted from the fruit epidermis, bark tissue of young branches and leaf material of infected and uninfected pear. The tested material was detached from the trees 24 to 48 hr prior to extraction. RNA extraction was carried out by "RNeasy Plant Mini Kit" manufacturer's protocol (Qiagen , www.qiagen.com , Cat No.74904).

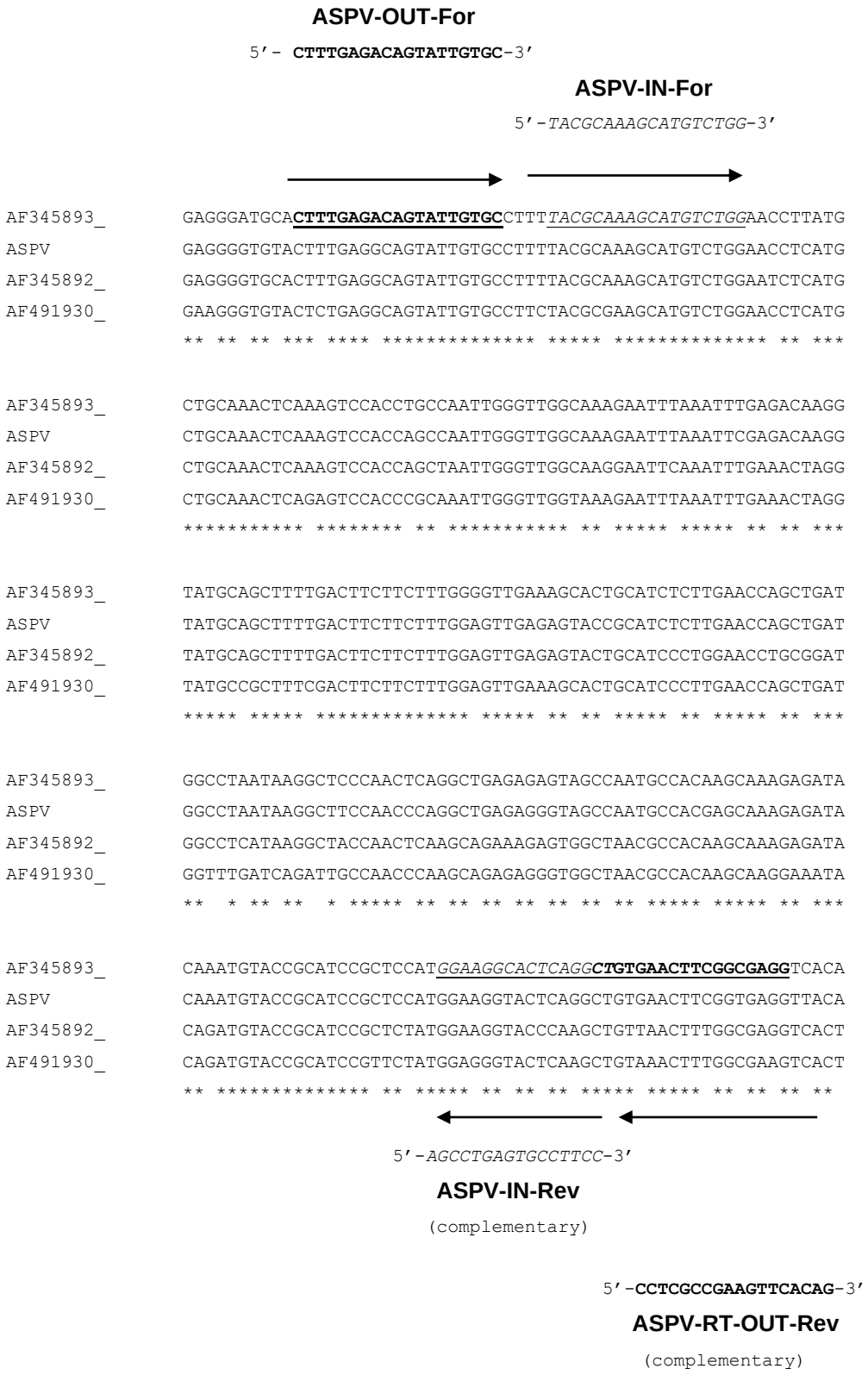


Figure 1. Primers designed for PCR of stony pit virus, and their position in relation to published ASPV coat protein DNA sequences.

PCR procedure

The first strand cDNA synthesis and the first round of amplification were carried out using *Tth* DNA polymerase –based "One Step RT PCR kit" **(**Novagen, Cat.No. 1089-3) with the oligonucleotide primers ASPV-RT-OUT according to the manufacturer's protocol.

The second PCR was carried out with Taq DNA polymerase (Qiagen, Cat No. 201203) by using 2.5 µl of the RT-PCR in a 25µl reaction volume and oligonucleotide primers ASPV-RT-IN. PCR conditions were: 94^{O}C – 3 min, then 35 cycles of (94^{O}C – 30 sec, 49^{O}C – 45 sec, 72^{O}C – 45 sec), and a final step of 72°C for 5 minutes.

The products of both PCRs were analysed by agarose gel electrophoresis on 1.4% gels run at 100-150V.

Validation of PCR

Two pear samples with suspected stony pit disease were submitted by growers and tested using the above protocol.

Grower survey

In order to determine incidence of suspected pear stony pit in the UK, and to provide samples for PCR testing, a survey form was devised and sent out to growers in July 2003. The questionnaire was designed to take the minimum of time for growers to complete and contained photographs of the problem to aid recognition.

A sample form is shown below (Figure 2).

Collection of field samples

In August 2003, six sites (total of nine orchards) were selected, based on a suspected pear stony pit incidence of 10% or greater. Tissue samples were collected and returned to laboratory for molecular analysis.

The sites locations were:

Kent (5 orchards). Total of 109 trees

Suffolk. Total of 10 trees

East Sussex. Total of 10 trees

Worcs.Total of 10 trees

Oxon. Total of 5 trees

At the Kent site, 30 trees were sampled from each of three orchards, planted in 1968, 1976, and 1994. Five were selected as positive controls, showing clear symptoms that could be attributed to stony pit disease. The remaining 25 were chosen using a stratified random sampling approach. A clean blade was used to excise samples from each tree. Samples were kept cool and RNA extracted within 24 hours. RNA was then stored at -80°C until required.

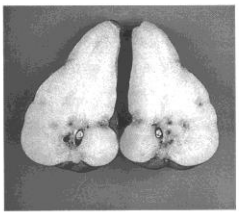
HDC SURVEY

Pear stone pit virus disease

1. Packhouse site

2. Have you graded out any fruit showing these symptoms in recent years? (see photograph above)

Yes	No



3. What % losses did you experience?

<5	6-10	11-15	16-20	>20

4. We would be grateful if you could provide further details below to enable us to contact you quickly to obtain samples for testing if needed

Office phone:	
Mobile phone:	
email address:	

Thank you for your help in completing this form.

Please return this form to the HDC in the pre-paid envelope by Monday 11th August 2003.

Figure 2 Grower survey form to determine incidence of stony pit virus

Validation of diagnostic test

Extraction of RNA and PCR amplification was performed as previously described. All samples collected were tested by PCR. Samples were run on agarose gels, positive bands excised, and then cleaned with a Qiaex band purification kit (Qiagen, UK), before sending to an external DNA sequencing service (Advanced Biotechnology Centre).

Redesigned primers and re-testing

As a result of the data obtained it was decided that the PCR primers were too specific and were unable to detect some isolates of pear stony pit. New primers were designed against ASPV (sequence AF495382) and their position is indicated in the table below compared to the original primers used in this project.

Table 1. PCR primers used in the project

Primer sequence	Usage	Position compared to Genbank AF345893 (coat protein gene)	Position compare to Genbar AF495382
CTTGAGACAGTATTGTGC	Original 1st rou forward	795-812	
CTGTGAACTTCGGCGAGG	Original 1st rou reverse	1078-1063	
GGNTGYACNYTNMGNCA RTAYTGYGC	New forwar	787-812	850-875
ACYTCNCCRAARTTNACN GC	New reverse	1079-1060	1142-1123

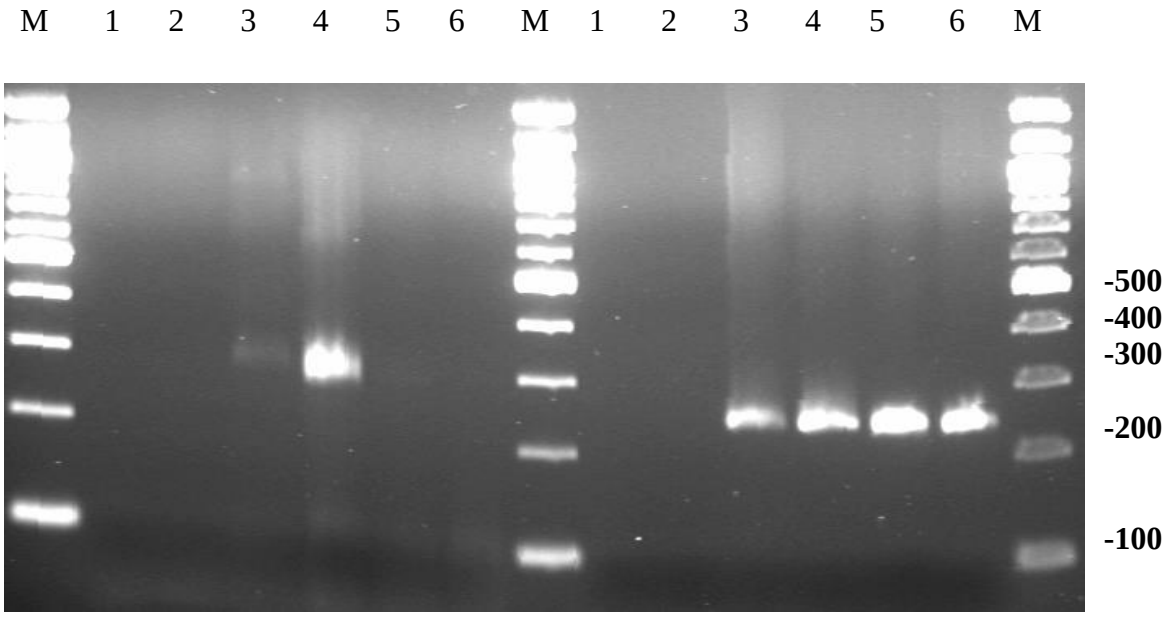
These new primers were degenerate, and comprised many possible actual sequence combinations, thereby increasing the chances of detecting a range of pear stony pit isolates.

PCR reactions were set up as for previous primer combinations. Selected positives were sent for sequencing to confirm that amplified products were stony pit virus. Samples were cleaned from gels or unloaded PCR product using a GFX Band Purification kit (AmershamPharmaciaBiotech). A minimum of 20ng/ul was then supplied with 12.8pmol forward primer for sequencing at the Advanced Biotechnology Centre, London.

Results and Discussion

Validation of initial primers and confirmation of stony pit relationship to ASPV

The specific band expected with the nested PCR procedure was detected only in the case of samples isolated from pear trees showing stony pit symptoms (2 out of 2 plants). No amplification products were detected in the case of samples isolated from the healthy pear trees (Figure 3). This indicated that pear trees with stony pit symptoms from the HRI collection contained an ASPV strain(s) closely related to the previously sequenced ASPV strains and that stony pit symptoms in these trees correlated with ASPV presence.



1- Non-infected pear, HRI collection (#1)
2- Non-infected pear (#2)
3- Stony pit pear, HRI collection severe symptoms (#1)
4- Stony pit pear, HRI collection, severe symptoms (#2)
5- Sample from grower, pear, mild stony pit symptoms (#1)
6- Sample from grower, pear, mild stony pit symptoms (#2)

Figure 3. Results of testing for stony pit virus in positive and negative controls, and suspected cases. Outer PCR primers were named 216 and 219, inner ones 217 and 218

Two pear samples with suspected mild stony pit disease, submitted by a grower, were tested. Both samples proved ASPV positive, although the level of ASPV after the first round of amplification was lower than in reference material showing severe symptoms, indicating the need for a second round of amplification (Figure 3).

Incidence of stony pit symptoms in UK orchards

Of the 214 survey forms sent to pear growers 128 were returned, a response rate of 59.8%. In comparison, the response rate to the Defra 2003 Orchard Fruit Survey was 72%. Of those who responded to this survey on stony pit disease, 57.0% reported some level of infection with stony pit disease in their pear orchards, but only 4.7% reported greater than 6% of trees affected (Table 2). The area reported with more than 6% of trees affected was 3.9 ha or 2.15% of the total area reported (Table 2). The total area of commercial pears registered with the HDC is 1,931.5 ha. If the level of affected trees reported by respondents to the survey were typical of the whole UK pear area then 41.5 ha would suffer an incidence of more than 6% of trees affected.

Table 2. Respondents Reports of Incidence of Stony Pit Disease in their Pear Orchards 2003

Incidence (%)	Number of respondents	% of respondents	Area (ha)	% of area reported	Infected area (ha)
None	55	42.97	256.4	30.48	
<5	67	52.34	566.7	67.37	14.2
6 to 10	0	0.00	0.0	0.00	0.0
11 to 15	3	2.34	1.4	0.17	0.2
16 to 20	2	1.56	14.4	1.71	2.5
>20	1	0.78	2.3	0.27	1.2
Total	**128**	**100**	**841.2**	**100**	**18.1**

Defra data (Basic Horticultural Statistics 2002/03) suggests an average pear crop value of £6,973 per hectare. Based on the HDC area and Defra crop value the total pear value for 2002/03 was some £13,468,349. If 2.15% of the area were affected then stony pit disease may be costing the UK pear Industry some £289,570 per annum.

The level of infection was reasonably consistent between different regions with the exception of Sussex and the West Midlands (Herefordshire; Worcestershire, Gloucestershire) where the proportion of trees affected appeared to be significantly higher. (Table 3). This may reflect the ages of pear orchards in these areas.

Table 3. Variation in Incidence of Stony Pit Reported by Respondents with Region

	Number of farms affected				Area affected (ha)		
County or area	**Yes**	**No**	**Total number**	**% affected**	**Yes**	**No**	**% affected**
Kent	39	28	67	58.33	415.2	224.2	64.94
Cambs	9	12	21	42.86	72.5	17.5	80.56
Essex	5	4	9	55.56	11.7	5.0	70.06
W Midlands	8	3	11	72.73	44.8	1.8	96.14
Sussex	4	1	5	80.00	5.9	0.3	95.16
S & West	2	2	4	50.00	6.0	1.6	78.95
Suffolk/Norfolk	3	3	6	50.00	12.7	5.6	69.4
North	3	2	5	60.00	16.0	0.4	97.56
Total	**73**	**55**	**128**	**57.03**	**584.8**	**256.4**	**69.52**

Anecdotal information from respondents suggested that most growers rarely saw symptoms as severe as those depicted in the photographs that accompanied the questionnaire. Some reported that symptoms were fewer and less severe in 2003 than in some seasons and that there was considerable variability from season to season. For some growers 2002 was a bad season for stony pit with high levels of incidence in fruit. This seasonal variation makes it exceptionally difficult for growers to have an impression of how severe the problem might be in their orchards and how the disorder may be developing through an orchard. Symptom expression may be linked to temperature or general growing conditions.

There was an indication that variety had little influence as incidence was reported in Conference, Comice, Concorde and Beurre Hardy. Comments which were made indicated that most growers regarded losses as insignificant, although some growers may not have been looking for the problem until stimulated to do so by the survey.

There is a small number of growers for whom the incidence of the disorder is clearly of significance in some years. Growers generally responded to severe infection by grubbing trees. The disorder was reported in both old and young trees. Some growers indicated that infection started at the end of rows.

A confounding factor was the similarity in symptom expression between stony pit disease and boron deficiency. The transient nature of symptom expression of stony pit may have added to this confusion as growers may have applied boron foliar sprays in some cases where stony pit was the casual agent of the disorder. Subsequent growing conditions may not have been conducive to symptom expression and thus growers felt that a "cure" had been achieved even if that were not the case.

It is also possible that the lack of severe symptoms being reported by respondents coupled with the random nature of expression may have caused "mild" infection and symptoms of the disorder to be overlooked or attributed to other causes of malformation and uneven shape in pear fruit.

PCR detection with first primer design

Using the primers described above, initial data was good – the primers amplified viral products from trees with severe and mild symptoms, and also from some trees that appeared healthy. Sequencing of six isolates revealed three different ASPV isolates.
However, PCR of further samples indicated that some trees that were positive for symptoms were proving negative by PCR. The number of these failures was sufficient to lead to the conclusion that these primers were too specific and so failed to detect the variation in some ASPV strains.

Validation of re-designed primers for ASPV

The redesigned primers were selected to detect as many isolates of stony pit virus as possible. PCRs performed on over 140 samples collected at orchards throughout the UK indicated that a single round of PCR was sufficient to amplify visible product of the expected size with the 'outer' primers only. Table 4 indicates that of all samples tested, primers match symptoms 45% of the time, detect virus in plants without symptoms 35% of the time and fail to match symptoms on 20% of plants.

Table 4. Symptoms attributed to stony pit virus on pear collected from several UK orchards and results of PCR tests on these samples

Samples	Symptoms		PCR positives
Site 1	None	58	34
	Mild	14	6
	Severe	27	13
Site 2	None	5	3
	Mild	0	0
	Severe	5	4
Site 3	None	5	3
	Mild	0	0
	Severe	5	3
Site 4	None	NC	2
	Mild	NC	Of
	Severe	NC	5
Site 5	None	5	3
	Mild	0	0
	Severe	5	3
Site 6	None	5	5
	Mild	0	0
	Severe	5	3
Positive controls	None	0	0
	Mild	0	0
	Severe	2	1

Overall incidence and PCR detection in plants without symptoms was much higher in younger trees at one particular site than older ones as shown in Table 5.

Table 5. Proportion of plants detected by visual assessment or PCR for stony pit virus from orchards of different ages at a single site (note fewer samples for 1972 may affect percentages compared to other years)

Site 1 – year orcha planted	Number of samples	No symptoms PCR positive (%)	No symptoms PCR negative (%)	Symptoms PCR positive (%)	Symptoms PCR negative (%)
1968	30	29	31	11	29
1972	9	33	11	45	11
1976	30	24	38	14	24
1994	30	59	14	0	27

The redesigned primers gave faint products only with some samples, but a second round of amplification led to multiple band generation making assessment of results difficult.

There were also samples with severe stony pit symptoms that gave negative PCR results, whilst one positive control (of 2) also failed to give a product.

Reasons for the apparent inability to PCR virus from plants with symptoms could include:

- Symptoms were not due to stony pit virus
- The PCR is not detecting all strains of the virus
- Viral distribution in tissues leads to sampling failure (i.e. tissue collected does not contain virus)
- The PCR is not detecting low virus titre
- PCR inhibition or viral degradation in storage

Sequencing to confirm the identity of the positives as pear stony pit disease was unsuccessful. This may be due to inadequate clean-up of samples, or to the presence of non-specific amplification products interfering with the sequencing reaction. Cloning into a plasmid vector would be required to resolve this with certainty.

Conclusions

PCR and sequence data from this project provide substantive evidence, but not firm proof, that ASPV is responsible for stony pit disease of pear. However, the variable nature of the virus has meant that development of a reliable molecular test for the disease has not proceeded to the stage at which a commercial diagnostic service can be offered.

Future work should take into account:-

- A nested approach to the PCR with redesigned primers to address the possibility of low virus titre leading to false negatives.
- Several samples should be tested from infected plants (at least three) to account for uneven distribution of the virus.
- By combining the original and redesigned primers it should be possible to ascertain whether sequence variability is causing difficulties with detection, and it may be possible to combine primer sets to counter this potential problem.
- The PCR conditions may be refined to improve detection.

Recent publications describe the use of PCR-ELISA and real-time PCR for detection of ASPV and of multiple apple viruses (Menzel *et al*., 2003; Salmon *et al*., 2002). Given the link between ASPV and stony pit virus such approaches should have value in diagnosis in pear.

The grower survey indicated that, although pear loss from stony pit disease is regarded as insignificant by most growers, it could account for losses in value of the UK pear crop of nearly £300,000 per annum. With a third of surveys not returned, the confusing factor of boron deficiency and the variable nature of symptom expression, the significance of the problem may be understated by these results. This indicates that there is a requirement for a rapid test to improve on the cumbersome graft test procedure, and also to facilitate studies into the apparent spread on virus into and within young orchards where healthy propagation material has been planted.

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