Project title:	The incidence and etiology of pear stony pit disease
Project number:	TF 130 (APRC SP130)
Report:	Year 1 annual report, June 2003
Project leader:	Dr Eugene Ryabov Horticulture Research International East Malling West Malling Kent ME19 6BJ
Key workers:	Kathy Pike
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Contents

Grower Summary

Background and expected deliverables

Fruit affected by stony pit are made worthless by pits on the surface and hard, stony lumps in the flesh of the fruit, which can also be severely misshapen (see Fig A). Symptoms vary in severity between seasons affecting from just a few to most of the fruit on a tree.

Fig A Typical symptoms of pear stony pit disease on mature pear fruit

In recent years suspected samples have been noted in 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 have been seen on cultivars Beurre Hardy, Conference, Doyenne du Comice and Concord.

The disease is believed to be 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. As symptoms are often attributed to boron deficiency, diagnosis can be uncertain without a confirmatory graft test. Graft-testing is usually impractical as it takes at least 3 years to complete.

It is not known how common the disease is and the aim of the project over a 2 year period is to:

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

ii) Gather information on factors affecting the incidence of Pear Stony Pit disease in commercial orchards

iii) Establish whether there is a strong correlation between Pear Stony Pit disease and Apple Stem Pitting Virus

iv) Provide guidance on disease prevention and control from the best available information

Summary of results and main conclusions (year 1, 2002)

The first aim was to provide a molecular diagnostic test for apple stem pitting virus (ASPV), the most probable cause of pear stony pit disease.

The test for ASPV was developed and validated (see further details in the science section), showing up as positive for pear trees from the HRI collection with known pear stony pit disease and also two samples from commercial orchards showing mild disease symptoms. This test is highly specific for this virus and adaptable to detect all characterised strains, with a possibility to detect previously uncharacterised ASPV strains.

Further work (year 2, 2003)

Work in year 2 (2003) will involve a grower survey to establish the incidence and economic importance of pear stony pit disease in the UK and to gather information on factors affecting disease incidence. Further tests will be conducted in leaf and fruit samples from infected and non-infected trees to establish the link with apple stem pitting virus (ASPV). The results of the project will provide guidance to growers on the possible causes of pear stony pit disease and options for prevention and control. Further work may be necessary thereafter.

Science Section

Background and objectives

Fruit affected by stony pit are made worthless by pits on the surface and hard, stony lumps in the flesh of the fruit, which can also be severely misshapen. Symptoms vary in severity between seasons affecting from just a few to most of the fruit on a tree.

In recent years suspected samples have been noted in 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 have been seen on cultivars Beurre Hardy, Conference, Doyenne du Comice and Concord.

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It is not known how common the disease is and the aim of the project over a 2 year period is to:

i) Determine the incidence and economic importance of Pear Stony Pit disease in commercial orchards in the UK

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iv) Provide guidance on disease prevention and control from the best available information

Materials and Methods

Detection of *Apple stem pitting virus* (ASPV).

To assess sensitivity and specificity of the test procedure, ASPV was tested for in two pear plants showing severe stony pit symptoms (for which ASPV presence was previously confirmed) and in two healthy pear plants. These plants were all from the HRI collection. Samples from each plant comprised bark from young branches, fruit epidermis, and leaf material. RNA was extracted from 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" following the manufacturer's protocol (Qiagen , Cat No.74904). This RNA extraction method does not include phenol and chloroform extraction and is highly reproducible. Primers for ASPV detection were designed by analysing the coat protein gene sequences of the four characterised ASPV isolates available in June 2002.

The first strand cDNA synthesis and the first round of amplification was carried out using *Tth* DNA polymerase –based **"One Step RT PCR kit"** (Novagen, Cat.No. 1089-3) with the oligonucleotide primers **#216** and **#219** according to the manufacturer's protocol. The second PCR was carried out with the **Taq DNA polymerase** (Qiagen, Cat No. 201203) by using 2.5 □l of the RT-PCR for 25□l reaction volume and oligonucleotide primers **#217** and **#218**. PCR conditions +94^{O}C – 3 min, then 35 cycles: 94^{O}C – 30 sec, 49^{O}C – 45 sec, 72^{O}C – 45 sec.

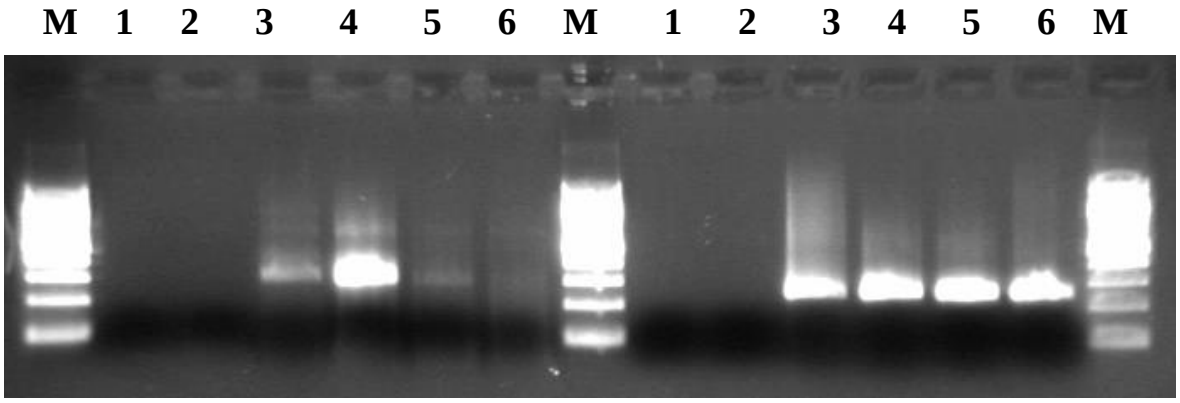
The products of both PCRs were analysed by agarose gel electrophoresis. In some cases the virus specific PCR product was detected after the first round of PCR (especially in the case of the reference plants with severe symptoms) while in suspected plants the amount of product was much lower. For that reason, the second round of PCR was needed in order to confirm the first-round positive findings.

Primers

			Position in ASPV the sequence Accession No AF345893 (Coat Protein gene)
216	CTTTGAGACAGTATTGTGC	**ASPV-OUT-For**	795 – 812, Forward
217	TACGCAAAGCATGTCTGG	**ASPV-IN-For**	817 – 834, Forward
218	AGCCTGAGTGCCTTCC	**ASPV-IN-Rev**	1047 – 1062, Reverse
219	CCTCGCCGAAGTTCACAG	**ASPV-RT-OUT-Rev**	1063 – 1078, Reverse

Results and Discussion

The products from the PCR tests were analysed by gel electrophoresis and the specific band expected was detected only in the case of samples isolated from pear plants showing stony pit symptoms (2 out of 2 plants). No amplification products were detected in the case of samples isolated from the pear plants which did not show stony pit symptoms (Figure 1 below).



Product 285 nt

First round of RT PCR
Primers 216 and 219

Product 246 nt

Second round of PCR
Primers 217 and 218

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 1 – Results of the PCR tests for ASPV in pear samples showing no symptoms (control – lanes 1 & 2), severe symptoms (lanes 3 & 4), and mild symptoms (lanes 5 & 6) of pear stony pit disease

In the first detection attempt (to the left of the middle marker (M)), a clear white band is seen in lanes 3 and 4. This band indicates a positive ASPV test for HRI samples. Lanes 1 and 2 remain blank – there is no ASPV in these. After further amplification (to the right of the middle marker (M)), the ASPV bands from the infected plants are enhanced, whereas lanes 1 and 2 remain dark, further confirming that ASPV was not present.

These results indicate that pear plants with stony pit symptoms from HRI's collection contain ASPV closely related to the previously sequenced ASPV strains.

The results also suggest that the test is highly specific and sensitive as well as being practical, robust and suitable for medium-scale screening of samples collected throughout Britain. RNA isolation, and downstream techniques require laboratory conditions and equipment, so samples would need to be sent to HRI-East Malling or an equivalent laboratory. Whilst validating the test, material collected from reference trees for several hours was kept deliberately at room temperature prior to RNA isolation to mimic the time delay to which samples could be subjected to when sent by post.

Two pear samples with suspected mild stony pit disease submitted by a grower were tested using the procedure outlined in this report. Both samples proved ASPV positive. The results as shown in Figure 1 indicate that a second detection phase (to the right of the middle M) was required to unambiguously confirm the presence of ASPV in the grower samples, because less ASPV was present (faint bands only in lanes 5 and 6 to left of middle M). Lanes 5 and 6 now show clear positive results similar to those in lanes 3 and 4. Again no ASPV was detected in the control samples isolated from healthy pears.

Although these results show a link between the presence of ASPV in pear and stony pit symptoms, it is not known how common the disease is in commercial orchards. In addition, information on strains of ASPV is fragmented and sparse so there is a need to collect additional information on the co-occurrence of ASPV and trees with pear stony pit symptoms. This work will be undertaken in year 2 of the project.

Appendix 1

Primer design

Primers for ASPV detection were designed by analysing the coat protein gene sequences of the four characterised ASPV isolates available in June 2002.

ASPV-OUT-For
5'- **CTTTGAGACAGTATTGTGC**-3'

ASPV-IN-For
5'-*TACGCAAAGCATGTCTGG*-3'

AF345893_
GAGGGATGCA**CTTTGAGACAGTATTGTGC**CTTT*TACGCAAAGCATGTCTGG*AACCT
TATG
ASPV
GAGGGGTGTACTTTGAGGCAGTATTGTGCCTTTTACGCAAAGCATGTCTGGAACCT
CATG
AF345892_
GAGGGGTGCACTTTGAGGCAGTATTGTGCCTTTTACGCAAAGCATGTCTGGAATCT
CATG
AF491930_
GAAGGGTGTACTCTGAGGCAGTATTGTGCCTTCTACGCGAAGCATGTCTGGAACCT
CATG
** ** ** *** **** ********** ***** ********** ** ***

AF345893_
CTGCAAACTCAAAGTCCACCTGCCAATTGGGTTGGCAAAGAATTTAAATTTGAGACA
AGG
ASPV
CTGCAAACTCAAAGTCCACCAGCCAATTGGGTTGGCAAAGAATTTAAATTCGAGAC
AAGG
AF345892_
CTGCAAACTCAAAGTCCACCAGCTAATTGGGTTGGCAAGGAATTCAAATTTGAAACT
AGG
AF491930_
CTGCAAACTCAGAGTCCACCCGCAAATTGGGTTGGTAAAGAATTTAAATTTGAAACT
AGG
********** ******** ** ********** ** ***** ***** ** ** ***

AF345893_
TATGCAGCTTTTGACTTCTTCTTTGGGGTTGAAAGCACTGCATCTCTTGAACCAGCT
GAT

ASPV
TATGCAGCTTTTGACTTCTTCTTTGGAGTTGAGAGTACCGCATCTCTTGAACCAGCT
GAT
AF345892_
TATGCAGCTTTTGACTTCTTCTTTGGAGTTGAGAGTACTGCATCCCTGGAACCTGCG
GAT
AF491930_
TATGCCGCTTTCGACTTCTTCTTTGGAGTTGAAAGCACTGCATCCCTTGAACCAGCT
GAT
***** ***** ********** ***** ** ** ***** ** ***** ** ***

AF345893_
GGCCTAATAAGGCTCCCAACTCAGGCTGAGAGAGTAGCCAATGCCACAAGCAAAG
AGATA
ASPV
GGCCTAATAAGGCTTCCAACCCAGGCTGAGAGGGTAGCCAATGCCACGAGCAAAG
AGATA
AF345892_
GGCCTCATAAGGCTACCAACTCAAGCAGAAAGAGTGGCTAACGCCACAAGCAAAG
AGATA
AF491930_
GGTTTGATCAGATTGCCAACCCAAGCAGAGAGGGTGGCTAACGCCACAAGCAAGG
AAATA
** * ** ** * ***** ** ** ** ** ** ** ** ***** ***** ** ***

AF345893_
CAAATGTACCGCATCCGCTCCAT*GGAAGGCACTCAGG**CT***GTGAACTTCGGCGAGG
TCACA
ASPV
CAAATGTACCGCATCCGCTCCATGGAAGGTACTCAGGCTGTGAACTTCGGTGAGGT
TACA
AF345892_
CAGATGTACCGCATCCGCTCTATGGAAGGTACCCAAGCTGTTAACTTTGGCGAGGT
CACT
AF491930_
CAGATGTACCGCATCCGTTCTATGGAGGGTACTCAAGCTGTAAACTTTGGCGAAGT
CACT
** ********** ** ***** ** ** ** ***** ***** ** ** ** **

5'-*AGCCTGAGTGCCTTCC*-3'
ASPV-IN-Rev
(complementary)

5'-**CCTCGCCGAAGTTCACAG**-3'
ASPV-RT-OUT-Rev
(complementary)