Project title:	The significance of orchard inoculum in apple canker epidemiology
Project number:	TF 36 (ii) [Previously APRC SP 36]
Report:	Final report 2001
Project leader:	T R Swinburne, Wye College
Key words:	apple canker, orchards, *Nectria galligena,* inoculum

This project report was originally issued by the Apple & Pear Research Council, under project number SP 36.

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MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

Research and Development

Final Project Report

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Project title	The significance of orchard inoculum in apple canker epidemiology
MAFF project code	**OC9518**
Contractor organisation and location	Horticulture Research International East Malling, West Malling Kent ME19 6BJ
Total MAFF project costs	£ 153,362
Project start date	01/07/98
Project end date	30/08/01

Executive summary (maximum 2 sides A4)

Apple canker is caused by the fungal pathogen *Nectria galligena*. The pathogen was considered to be spread within and between trees by rain-splashed and wind-borne spores. It was therefore assumed that disease development would follow the classic pattern of an aerially spread pathogen. However, in the late 1980s there was a spate of canker outbreaks, especially in young orchards of certain dessert apple varieties, cvs. Royal Gala and Fiesta. Often these trees had large multiple cankers on the rootstock or along the main stem. When stem sections were examined there was extensive staining of the xylem vessels. This, and other circumstantial evidence, suggested that the fungus might have means of spread other than by external inoculum. Indeed it seemed probable that *N. galligena* was able to spread systemically within trees and that the initial infection had occurred at the propagation stage. Trees, which became infected at this early stage, might remain symptomless and only develop canker up to three years after planting.

(Objective 1 - Achieved). At the outset of the project a DNA molecular marker system was developed to discriminate between different populations of the pathogen. This method was adopted and used throughout the rest of the project.

(Objective 2 - Achieved). A magnetic-capture hybridisation method was developed for the detection of *N. galligena* in wood. This method enabled the pathogen to be detected in asymptomatic wood and also in old cankers, which could not be achieved without magnetic-capture. However, because of the extreme sensitivity of the method it led to a significant number of false positive reactions from the control samples. Although it remains important to have a sensitive method of detecting *N. galligena* in wood, further development of magnetic-capture may not be the best approach. There are now improved proprietary DNA extraction procedures available, which could give more reliable results.

(Objectives 3 & 4 - Achieved). Using the DNA marker system that had been developed, population studies of *N. galligena* were carried out in three orchards where young trees had been planted in close proximity to older established trees or where areas of the orchard had been planted with trees originating from different nurseries. This showed that there was spread of *N. galligena* both within and between orchards. This conclusion of the occurrence of aerial spread was further confirmed in a trial to assess the tree to tree transmission of a unique *N. galligena* isolate of known genotype. Within the first season after establishment of cankers, the pathogen was shown to have spread within the infected trees and caused new cankers and fruit rots. As the trial progressed, there was evidence of spread to neighbouring trees. It was impossible, in this trial, to ascertain the full extent of the spread within the orchard.

(Objective 5 - Achieved). In some earlier trials, pre-dating this project, as well as in a Bramley's Seedling trial at NIHPBS, N. Ireland, the level of cankers developing on trees from different nursery sources was influenced by the source of the trees. Unfortunately, it was not possible to back up these results with molecular information. They do, however, strongly suggest that the amount of canker that develops after planting is influenced by factors associated with the nursery that supplies the trees.

A major trial was planted in 1998 (The Millennium Trial) which was designed to determine the relative importance of aerial spread as opposed to nursery infections. Three nurseries produced both rootstocks and budwood, cv. Royal Gala. They then exchanged this material and grafted the budwood from each of the nurseries on to rootstocks from each of the nurseries and grew on the resulting trees. This resulted in a total of twenty-seven treatments (3 budwood sources x 3 rootstock sources x 3 nurseries where trees were assembled). The trees were then planted at HRI - East Malling, ADAS - Rosemaund and NIHPBS – Loughgall in randomised blocks. Canker development was assessed in each orchard every autumn and spring, until spring 2001. Cankers were collected, their position on the tree having been recorded. Particular attention was given to cankers developing on the rootstock or main-stem (designated 'A' and 'B' cankers respectively). *N. galligena* was isolated from a selection of the cankers.

The pattern of canker development was different at each of the trial sites. At Rosemaund, which was remote from other orchards and hence external sources of canker, only a small number (82) of cankers developed. The numbers were too small to analyse statistically, although the few cankers developing on the rootstocks or mainstem (< 50) did suggest that the level of infection originating from the nursery was low (<5% of the trees planted).

In contrast, at NIHPBS, canker developed very rapidly. After three growing seasons over 70% of trees had at least one canker and in excess of 2000 cankers were recorded over the whole site during the course of the experiment. There was a strong positional effect on canker development within the orchard. Canker appeared to spread from the north-east corner of the orchard towards the south-west. On the northern side of the Millennium Trial there was a mature Bramley's Seedling orchard with significant levels of canker. It was therefore evident that, in situations of high inoculum and optimum disease conditions, aerial spread is the most important means of dissemination of this pathogen. When this positional effect was statistically removed there were significantly more cankers developing in trees which had been assembled at one of the nurseries, suggesting that some stage of the tree construction (e.g. grafting or heading back) may be an important time for infection.

At East Malling relatively few cankers developed until the final assessment, in spring 2001, when large numbers were recorded. This may have been due to an unusually wet winter predisposing trees to infection and symptom expression. As at NIHPBS, there was a significant positional effect but when this was removed significant differences were again seen between nurseries which assembled the trees, with the same nursery being associated with significantly more cankers.

Molecular analysis of isolates of *N. galligena* obtained from cankers collected from the Millennium Trial indicated that the population of the *N. galligena* associated with rootstock and budwood cankers was different from that associated with peripheral cankers. This suggests that a proportion of the infections on the rootstock and mainstem could have been introduced with the trees. The populations of *N. galligena* from peripheral cankers on trees in N. Ireland and Kent were distinct. Too few isolates were obtained from the Rosemaund site to permit conclusions to be drawn from either statistical analysis of numbers of cankers or molecular analysis of isolates. This further confirms the importance of external infections from neighbouring orchards.

We have demonstrated that:

- **Infections without symptoms could be detected by direct isolation, immunofluorescence and magnetic capture probes indicating that the pathogen may spread systemically without external symptoms.**
- **Inoculation experiments made at various stages of propagation indicated that infections occurring at grafting could remain dormant for up to 5 years.**
- **When planting new orchards some infection may be brought in with the trees.**
- **Cankers on the main stem may be due to either nursery infections or infection in the orchard from external inoculum sources.**
- **The most probable time for nursery trees to be infected is at grafting.**
- **Aerial spread is very important in development of epidemics.**

Therefore, although some factors affecting infection and disease expression of apple canker remain unclear, the main conclusions of the project are
(i) some infection may be brought into new orchards on the trees but
(ii) the majority of infections in all but the most isolated sites will arise from spread from external sources.

This means that while control of infection in nurseries may reduce the numbers of the particularly damaging early infections on main stems these will not be entirely eliminated and control in the orchard must remain a major part of Nectria canker management.

Scientific report (maximum 20 sides A4)

INTRODUCTION

Apple canker is caused by the fungal pathogen *Nectria galligena*. The pathogen can infect wood of all ages resulting in cankers. Where these develop in side shoots the damage can be relatively insignificant. Infections can be pruned out and good control can be achieved through autumn and spring applications of fungicides (Cooke *et al.* 2000). It had been considered that such cankers arose from aerial spread conidia of *N. galligena* which infected leaf and bud-scale scars or other wounds (Swinburne 1975). Rain is an important vector of the pathogen both by aerial rain-splash from tree to tree and rain-splash and run off within trees. *N. galligena* can also initiate fruit rotting which may be severe in certain seasons.

In the late 1980s there was a spate of severe canker in young orchards, particularly on new cultivars such as 'Fiesta' and 'Gala'. Often these young trees had developed large multiple cankers near the base or along the main stem. When stem sections of infected trees were examined there was extensive staining and hyphae of a type common to *Nectria* in the xylem. This evidence suggested that that there may be means of spread and infection other than external inoculum and that the pathogen may be able to spread systemically within trees. The idea of systemic infection was further supported by the fact that in a number of commercial orchard planted in areas remote from significant sources of inoculum trees succumbed to canker, particularly in the first few years after planting. Furthermore individual trees could have multiple severe canker whilst their neighbours were disease free. This did not seem to tie in with a random eternal source. It was therefore hypothesised that the nursery may be a source of canker. It seemed possible that trees could become infected during the propagation stage, but remain symptomless until after planting, with canker not being evident for up to three years.

MAFF funded a project starting in 1996 which reported in 1998 (OC9518: 31.08.98) There was significant overlap with the current project in terms of objectives, hence this report will describe all five objectives common to both projects. The objectives as set out in the original proposal were:

OVERALL OBJECTIVE

To determine the importance of infection originating in the nursery to the development of canker epidemics, to identify the stage in the propagation process at which most infection in the nursery occurs, and from these assess the cost effectiveness of including canker in certification procedures.

The specific objectives to achieve these are:-

1. To devise DNA marker systems to discriminate genotypes of *N. galligena* in orchard and nursery populations.
2. To develop a sensitive, reliable and rapid method to detect the pathogen in cryptically infected material based on existing PCR techniques but avoiding the use of hazardous chemicals.
3. Using the selected DNA marker system (01) conduct a population study *of N. galligena* in commercial orchards to identify sources of infection.
4. Assess the rate of tree-to-tree transmission by setting up point sources of isolates of known genotype.
5. Determine the most frequent mode of infection and the source of inoculum within nurseries.

OBJECTIVE 1
To devise DNA marker systems to discriminate genotypes of *N. galligena* in orchard and nursery populations.

At the outset of the project as initially described, it had been intended to compare different approaches to discriminating fungal genotypes during the winter preceding the first summer season. However, this was not done for two reasons; firstly the project did not start until too close to the summer season to allow the time to do this and secondly it became clear that an appropriate working system was available.

Developed as part of the doctoral thesis project of Dr S.H. Langrell at Wye College, University of London (as it then was) the method adopted was based on a high level of variability present in the intergenic regions (IGR) of the ribosomal RNA gene repeats. This variability arises from three effects. Firstly (and possibly most importantly), *N. galligena* has, like many other fungi, sub-repeats within the IGR sequences which vary in number. This number variation gives rise to major differences in amplicon or restriction digest fragment length. Variation may also arise from insertions and deletions and from sequence changes affecting restriction endonuclease recognition sites. The overall variability is accessed by PCR using conserved primers designed to amplify the IGR, followed by restriction endonuclease digestion and agarose gel electrophoresis to visualise polymorphisms (IGR PCR-RFLPs). The main weakness of this approach is that because these primers are not species specific it is necessary that isolates to be tested are first got into pure culture. This requirement limits the number of samples that can tested. The development work for this approach has been described as part of a paper submitted to European Journal of Plant Pathology and will be described only briefly here. The full text of the submitted paper can be seen in appendix 1. For use in this project the method was simplified by using fewer restriction endonucleases as preliminary experiments had shown that using eight enzymes did not give more useful discrimination than did the four used here.

The IGR PCR-RFLP method comprised three stages. After the often difficult isolation of the fungus from the apple wood sample, DNA extracted from each isolate was amplified using the primers mentioned above, designated PN11/PN22 (see appendix for sequences) to give a product which varied in length depending on the isolate but which was in the range 2.5 to 3.0 kbp. The amplicons were then digested separately with each of the restriction endonucleases *Hae*III, *Hinf*I, *Hha*I and *Taq*I. The digestion products were then separated and visualised by standard agarose gel electrophoresis and the four patterns for each isolate compared with those for other isolates. Based on the patterns the isolate could then be classified into groups.

This method was used extensively in objective 5 for comparing isolates and populations of *Nectria galligena* in orchards.

OBJECTIVE 2
To develop a sensitive, reliable and rapid method to detect the pathogen in cryptically infected material based on existing PCR techniques but avoiding the use of hazardous chemicals.

The initial part of the work reported here formed part of the PhD thesis of Dr S. Langrell.

The internal transcribed spacers of the ribosomal RNA genes (ITS1 & 2, rRNA genes) are flanked by highly conserved sequences and hence are readily accessible using PCR and standard procedures. The ITS regions of 56 *N. galligena* isolates were sequenced and compared with the known sequences of closely related species. From this, primers were designed which were shown to amplify DNA only from *N. galligena* (producing a product of 412 bp) but not DNA of related species or some apple endophytes. These primers were sensitive and

reliable under many circumstances, including when using extracts of 'young', recently formed cankers. They were not however consistently effective for the detection of *N. galligena* directly in wood samples taken from asymptomatic areas from beyond regions of staining immediately adjacent to cankers nor in samples from 'old' very well-established cankers (data not shown). This lack of consistency was thought to be due to the presence of inhibitors extracted from the tissue.

To avoid the presence of inhibitors a novel approach to the purification of fungal DNA was attempted *viz.* magnetic capture hybridisation. This work has now been published (Langrell SRH & Barbara DJ (2001) "Magnetic capture hybridisation for Improved PCR detection of *Nectria galligena* from lignified apple extracts", *Plant Molecular Biology Reporter*, **19**, 5-11: also published on-line at http://www.nrc.ca/cisti/journals/ispmb/reporter.html as volume 19, issue 3) and full details of the results can be read in the copy appended to this report.

In brief, an 81 base pair oligomer, based on the ITS sequence between the described primers above, was synthesised and attached via a biotin moiety to *para*-magnetic beads. DNA extracts from woody tissues were prepared using a standard procedure, denatured by heating to 99° C and mixed with the bead/oligomer conjugate. Holding the mixture at 66° C for 4 hrs led to the *N. galligena* DNA hybridising to the capture oligomer. A simple washing procedure using a magnet to collect the beads/oligomer/DNA complex lead to the effective removal of contaminants. Using the primers described above, PCR was then effective and highly sensitive, down to a theoretical one DNA molecule and about 10-100 fold more sensitive than for PCR without capture. This extra sensitivity and removal of inhibitors allowed the detection of *N. galligena* in asymptomatic wood and in old cankers, which could not be achieved without magnetic capture (See paper for results).

Unfortunately, in routine use, this procedure has proved to suffer from a major drawback i.e. the extreme sensitivity leads to a significant proportion of false positive reactions amongst the control samples. Due to a lack of any further time allocated to this approach we have been unable to solve this problem. If there is still a need for a very sensitive detection system for *Nectria,* because of other developments it is not clear that this approach should be continued. There are now a number of improved proprietary DNA extraction procedures on the market and it may be better to investigate these alternative methods rather than continue with development of the magnetic capture hybridisation method.

OBJECTIVE 3

Using the selected DNA marker system (01) conduct a population study *of N. galligena* in commercial orchards to identify sources of infection.

Chartham Hatch and Pencoyd Trials

Orchard sites and canker sampling: Two orchards in Kent and one in Hereford were selected for the study. The two orchards in Kent were located on a farm at Chartham Hatch. The trees in orchard one (Hop Garden) consisted of cvs Cox and Gala of known nursery origin (Cox - nursery in Kent and Gala – nursery in France) and were less than five years old. The orchard was divided into 3 areas. Areas A and B were adjacent to an old (30+ years) pear and an apple orchard respectively; Area C was remote from external sources of inoculum. Sampling was carried out in January 1997 to collect cankers from old and young wood and in June 1997 to collect additional cankers on one year old wood that had developed from autumn leaf scar infections. Records were made of tree location, wood age and tree cultivar. In addition a random sample of cankers were collected from the adjacent pear orchard and apple orchard, which were of different nursery origin to the young orchard. The cankers collected were stored at –20°C prior to isolation.

Conclusions

- There was a difference in the levels of canker on Bramley's Seedling trees originating from different nurseries which gives some support to the hypothesis that infections can arise from a nursery source.

OBJECTIVE 4:

Assess the rate of tree-to-tree transmission by setting up point sources of isolates of known genotype.

An experiment was established at HRI in order to measure the extent and speed of tree to tree spread of *Nectria galligena* from a point source. Four trees in a mature Cox orchard containing rows of Discovery / Spartan were inoculated with unique (on the basis of molecular-finger printing) isolates of the pathogen. Inoculation was done by inserting a small piece of agar from a pure culture of *N. galligena* into a small cut made in the wood. The inoculated wound was covered with bandage-like material to prevent drying out before infection had occurred. Two trees towards the north of the orchard were inoculated in the summer 1998 with 'Isolate E8', a fruit isolate of *N. galligena* from HRI, East Malling. At the same time a further two trees, near the bottom of the orchard and well separated from the other inoculated trees, were inoculated with 'Isolate W60', a wood isolate obtained from Wye College.

Any cankers that developed in the inoculated trees, apart from the original inoculation, were collected during 1998 / 99 and *N. galligena* was isolated and typed. Fruit was also collected in 1998, 1999 and 2000 and *N. galligena* isolated from any eye rots which developed during storage.

Fruit was collected from:

a) Around the initial inoculation site
b) On the same tree as the initial inoculation site
c) From trees immediately surrounding the inoculated tree

In the first year after inoculation (1998) rotting only occurred in fruit taken from immediately around the original inoculation. In the second year (1999) ca. 200 isolates were obtained from rotting fruit obtained from the inoculated tree and adjacent trees. Isolate E8 was re-isolated from rotting fruit obtained from trees close to the site of the E8 inoculation and similarly isolate W60 was obtained from fruit adjacent to the W60 inoculation. This result confirms the ability of the pathogen to be disseminated from an active canker within an infected tree and also to spread to neighbouring trees.

OBJECTIVE 5:

Determine the most frequent mode of infection and the source of inoculum within nurseries.

MILLENNIUM TRIAL

In 1996 three nurseries (**A** - Maurice Sarson, Tonbridge, Kent: **B**- Blackmore Nurseries, Liss, Hampshire and **C** - Frank P. Matthews, Tenbury Wells, Worcestershire) produced rootstocks (I, II, III), M9 (Blackmore obtained rootstocks from Pageon, France) and budwood (1,2,3,), Royal Gala (Blackmore obtained material from France, Mondial Gala). Each of the three nurseries exchanged rootstocks and budwood, which they duly grafted in spring 1997 at each of the nurseries, giving a total of 27 combinations ((**1**) A/I/1; (**4**) A/II/1; (**7**) A/III/1; (**2**) A/I/2; (**5**) A/II/2; (**8**) A/III/2; (**3**) A/I/3; (**6**) A/II/3; (**9**) A/III/3; (**10**) B/I/1; (**13**) B/II/1; (**16**) B/III/1; (**11**) B/I/2; (**14**) B/II/2; (**17**) B/III/2; (**12**) B/I/3; (**15**) B/II/3; (**18**) B/III/3; (**19**) C/I/1; (**22**) C/II/1; (**25**) C/III/1; (**20**) C/I/2; (**23**) C/II/2; (**26**) C/III/2; (**21**) C/I/3; (**24**) C/II/3; (**27**) C/III/3).

Over 3000 trees in total were produced. They were divided into three batches, which were sent to HRI, East Malling, ADAS, Rosemaund or NIHPBS, Loughgall for planting. Trees were planted in Spring 1998 in eight

tree plots. However because of some losses during the propagation stage a small number of plots at each site had less that eight trees. Plots were fully randomised over five blocks (four at Rosemaund). The ADAS, Rosemaund site was in an area isolated from any major apple planting and hence exposed to a low or negligible level of *N. galligena* inoculum. The East Malling orchard was close to other orchards, some of which had canker. The orchard in Northern Ireland was similarly close to both experimental and commercial orchards, particularly in the north and east. These orchards had relatively high levels of canker. Furthermore the wet weather conditions in N. Ireland are particularly conducive for the aerial spread and development of canker. During the summer 1998, three months after planting, trees at all three sites were assessed on a 1 – 3 scale for vigour (2 = normal: 1 = smaller: 3 = larger). The assessment of vigour was done to determine if trees from a particular nursery were larger or smaller than the other two.

Starting in autumn 1998 trees were assessed for canker twice a year – in the late autumn and early spring. The position of each canker was noted (A= rootstock: B = mainstem: C = at point of heading back: D = side shoot / main stem: E = everything else). The age of the wood was also noted. Cankers were removed and stored in the deep freeze after which time an attempt was made to isolate *N. galligena*. Where cankers had developed on the rootstock or mainstem this resulted in the complete removal of the tree. A selection of isolates was then typed using the molecular methods developed under Objective 1. Particular attention was paid to the 'A' and 'B' type cankers. It had been argued that it was possible that these cankers may have arisen from a nursery infection and may therefore be different from 'C', 'D' and 'E' cankers, which had been the result of external inoculum.

Total numbers of cankers per tree were recorded and statistically analysed for differences between treatments, i.e. nursery source of rootstock, nursery source of budwood and nursery where the tree was assembled and grown on. Again a distinction was made between 'A' and 'B' type cankers and 'C', 'D' and 'E' type cankers. In order to assess the effects of treatments on canker development it was necessary to allow for any strong positional effect that may have occurred at any of the sites. The degree of clustering at each site was investigated by plotting the cumulative numbers of cankers recorded for each tree at various times during the course of the trial on a 2-D representation of the site.

Development of canker at each site (A full statistical report is appended – Appendix 1)
Tree girth, summer 2001: There was considerable variation in tree girth between sites. The largest trees were at the N. Ireland site and the smallest trees at HRI, East Malling. The only consistent treatment effect across all sites was the significantly (P=0.05) smaller girths recorded from Nursery B relative to nurseries A and C which did not differ significantly (Tables 1a – c).

Table 1a. Mean tree girth (cm) at HRI, East Malling in summer 2001 (i.e. after 3 years in the orchard) categorised by nursery producing the rootstock, budwood or assembling the whole tree.

Assembled		Bud-wood			Total
	Root-stock	1	2	3	
A	I	8.9	9.0	9.0	**9.0**
	II	8.6	8.0	9.0	**8.6**
	II	9.3	9.0	8.9	**9.1**
	Total	**8.9**	**8.7**	**9.0**	**8.9**
B	I	8.1	8.4	7.9	**8.1**
	II	7.6	7.8	8.1	**7.9**
	III	8.0	8.2	8.4	**8.2**
	Total	**7.9**	**8.2**	**8.1**	**8.1**
C	I	8.8	8.5	9.0	**8.8**
	II	8.4	8.2	8.5	**8.4**
	II	8.7	8.4	8.9	**8.6**
	Total	**8.6**	**8.4**	**8.8**	**8.6**

Table 2 b. Mean tree girth (cm) at NIHPBS Loughgall, NI in summer 2001 (i.e. after 3 years in the orchard) categorised by nursery producing the rootstock, budwood or assembling the whole tree.

Assembled		Bud-wood			Total
	Root-stock	1	2	3	
A	I	10.2	10.0	9.9	**10.0**
	II	11.1	10.2	10.0	**10.4**
	II	10.2	10.1	10.2	**10.2**
	Total	**10.5**	**10.1**	**10.0**	**10.2**
B	I	9.5	9.7	9.6	**9.6**
	II	9.7	9.6	9.9	**9.8**
	III	10.1	10.2	9.4	**9.9**
	Total	**9.8**	**9.9**	**9.6**	**9.8**
C	I	10.3	10.4	10.6	**10.4**
	II	10.3	10.2	11.1	**10.5**
	II	10.1	10.7	10.0	**10.2**
	Total	**10.2**	**10.4**	**10.6**	**10.4**

Table 1 c. Mean tree girth (cm) at ADAS, Rosemaund, NI in summer 2001 (i.e. after 3 years in the orchard) categorised by nursery producing the rootstock, budwood or assembling the whole tree.

Assembled	Root-stock	Bud-wood			Total
		1	2	3	
A	I	9.8	9.9	10.2	**10.0**
	II	10.4	10.0	10.0	**10.1**
	II	9.5	1.4	10.2	**10.0**
	Total	**9.9**	**10.1**	**10.1**	**10.0**
B	I	9.7	10.2	9.5	**9.8**
	II	8.9	8.6	9.4	**9.0**
	III	9.3	8.9	9.9	**9.3**
	Total	**9.3**	**9.2**	**9.6**	**9.4**
C	I	9.2	9.9	10.1	**9.7**
	II	9.2	9.5	9.7	**9.5**
	II	9.9	10.1	9.5	**9.8**
	Total	**9.4**	**9.8**	**9.7**	**9.7**

(i) Rosemaund: By the final assessment date a total of 82 cankers had been recorded at the Rosemaund site. These represented 45 'A' and 'B' type cankers and 37 'C', 'D' and 'E' type cankers. These numbers were too small to show any statistically significant differences between the number of cankers or any type on trees from each of the nursery combinations. Similarly canker numbers at Rosemaund were too low to visually draw any conclusions regarding positional effects. However, the appearance of 45 rootstock and mainstem cankers at this site suggested that the maximum level of nursery infection was low (<5%). Such a level may be important to the grower as even this level of loss may be unacceptable, particularly where outside inoculum is low but in situations of high external inoculum it may not be significant.

(ii) NIHPBS, Loughgall, Northern Ireland: Canker was first observed in the trees at NIHPBS within 6 months of planting. The numbers of 'C', 'D' and 'E' type cankers rose rapidly so that by November 2000 over 70% of trees had at least on canker on the peripheral parts. 'A' and 'B' type cankers were obvious by February 1998 with a gradual increase at each assessment date. On the last assessment date in November 2000 almost 20% had cankers of this nature. There were strong positional effects for both canker groups. Canker appeared to spread from the north east corner towards the south-west. This pattern of spread would tie in with both the source of inoculum from neighbouring orchards and from wind direction (Due to their large size and number all figures showing spatial distribution of cankers in each of the three nurseries are presented in the statistical analysis of the Millennium Trial in the attached appendix).

The most consistent treatment effect on canker number was that observed for the nursery which assembled the tree by putting the rootstock and budwwod together. This can be illustrated by calculating the ratio of the number of cankers, of all types, in trees from nursery A to the number of cankers from nursery C. This ratio was 1.3, suggesting that trees assembled at nursery A were 1.3 times more likely to get cankers irrespective of the position of the tree in the orchard. There being no significant difference in the girth of trees from these nurseries would suggest that these differences were not due to differences in tree size.

(iii) HRI, East Malling: Canker was first observed in the trees at HRI East Malling in August 1998. The total number of cankers of all types was very much smaller that at NIHPBS. By August 2000, 169 cankers had been recorded on approximately 9% of the trees in the orchard. Of these cankers 54 were 'A' and 'B' types while 115 were 'C', 'D' and 'E' types. There were significant differences between sub-rows for groups 'A' and 'B' (P=0.026) and groups 'C', 'D' and 'E' (p=0.007) suggesting a non-random spread of cankers across the site. These strong positional effects were still obvious at the last assessment date in summer 2001.

Once again the nursery where the trees were assembled had a significant effect on canker numbers (P=0.001) for types 'A' and 'B'. None of the treatments (nursery sources) had significant effects on 'C', 'D' and 'E' type cankers.

An estimate of the relative importance of at NIHPBS and East Malling of the numbers of cankers originating from nurseries, as based on the development of A and B type cankers in the first two years, versus external spread is in the region of 1 –2 %.

Molecular analysis of *N. galligena* isolates from Millennium Trial: For molecular analysis, *N. galligena* was isolated from cankers as described previously and analysed by the same molecular method. Pure *in vitro* cultures were obtained for approximately 60% of the cankers for which isolation was attempted. Molecular analysis of isolates from NIHPBS trees was carried out at HRI-Wellesbourne; all other isolates were analysed at HRI-East Malling. Consequently the numbering of molecular types is not directly comparable (see below).

A total of 176 isolates representing approximately equal numbers of A/B and C/D/E cankers (see above for explanation of canker types) were molecularly typed (Table 2). As only a few isolates from ADAS-Rosemaund (16) and HRI-East Malling (37) were available for analysis these English samples were combined. For both the NIHPBS and HRI/ADAS sets, many of the isolates occurred infrequently (once or twice) and in only the A/B or C/D/E populations. This indicates that the all the populations are highly diverse according to this marker. This very high diversity means that these infrequent types cannot be used to compare directly the populations. However, sampling from a homogenous population ought to give similar distributions of the frequency of occurrence of different types. As can be seen from Table 2, the two sets of isolates from A/B cankers gave close to 3:1 ratios for types that occur only once to those occurring twice. Isolates from C/D/E cankers from NIHPBS gave a ratio closer to 2:1 and those from HRI/ADAS gave a ratio of over 4:1. This suggests the two sets of isolates from A/B cankers might represent one population whilst those from C/D/E represent two populations distinct both from each other and from the A/B canker isolates. The more frequently occurring types can be compared more directly. In the NIHPBS samples four types occur more than twice; type 8 represented 34% of the A/B isolates and 47% of the C/D/E isolates; type 2 represented 11% of the A/B isolates but did not appear in the C/D/E isolates; type 27 was 15% of the C/D/E isolates but only 1.5% of the A/B. Types 2 and 27 therefore clearly show these isolates to be distinct. Type 16 represented 6 and 4% of the two populations and did not distinguish them. Among the English isolates type 2 represented 37% of the A/B isolates but only 5% of the C/D/E isolates. The frequency of the NIHPBS type 8 in A/B cankers was very similar to that of the English type 2 in equivalent cankers and the digestion patterns of these two types were compared directly and found to be identical. (Based on frequency of occurrence several other possible matches

between NIHPBS and English types were postulated but when compared directly none were found to correspond.) Amongst the English isolates Type 26 and possibly 1 and 24 occurred more frequently in the A/B population than in the C/D/E one. Conversely types 3 and 20 and possibly 4 occurred more frequently amongst C/D/E isolates than among the A/B population.

Table 2. Listing of molecular types of *N. galligena* taken from rootstock/main stem (A/B) or heading back wound plus all other wood (C/D/E) from (a) NIHPBS (b) combined HRI-East Malling and ADAS-Rosemaund orchards established with trees and components from three sources (*i.e.* 'The Millennium Experiment'). Isolates were from cankers collected from various dates up to 2000.

a) NIHPBS.

Type*	Canker Class		Type	Canker Class		Type	Canker Class	
	A/B	C/D/E		A/B	C/D/E		A/B	C/D/E
2	**7(11)****	-	15	1(1.5)	1(2)	28	-	1(2)
3	2(3)	-	16	4(6)	2(4)	29	-	1(2)
4	1(1.5)	-	17	2(3)	-	30	2(3)	2(4)
5	1(1.5)	-	18	2(3)	-	31	1(1.5)	-
6	1(1.5)	-	19	1(1.5)	-	32	1(1.5)	-
7	2(3)	-	20	1(1.5)	-	33	-	1(2)
8	**22(34)**	**26(47)**	21	1(1.5)	-	34	1(1.5)	-
9	-	2(4)	22	1(1.5)	-	35	1(1.5)	-
10	-	1(2)	23	2(3)	2(4)	36	1(1.5)	2(4)
11	-	1(2)	24	1(1.5)	-	37	1(1.5)	1(2)
12	-	1(2)	25	1(1.5)	-	38	1(1.5)	-
13	-	1(2)	26	-	1(2)	39	1(1.5)	-
14	-	1(2)	**27**	**1(1.5)**	**8(15)**	Total Tested	64	55

b) HRI-East Malling/ADAS-Rosemaund

Type*	Canker Class		Type	Canker Class		Type	Canker Class	
	A/B	C/D/E		A/B	C/D/E		A/B	C/D/E
1	2(10)	-	10	-	1(3)	**20**	**1(5)**	**4(11)**
2	**7(35)**	**2(5)**	11	-	1(3)	21	-	1(3)
3	**1(5)**	**5(14)**	12	-	1(3)	22	-	2(5)
4	1(5)	3(8)	13	1(5)	1(3)	23	-	1(3)
5	-	2(5)	14	1(5)	-	24	2(10)	1(3)
6	-	1(3)	15	-	1(3)	25	1(5)	1(3)
7	-	1(3)	16	-	1(3)	**26**	**3(15)**	**1(3)**
8	-	1(3)	17	-	2(5)	27	-	1(3)
9	-	1(3)	18	-	1(3)	Total Tested	20	37

*Molecular categories are not comparable between NIHPBS and the other sites. ** Number of times (percent of total) a molecular type was found.

Despite the relatively low number of cankers analysed, these data show the isolates from A/B cankers collected in the first three years after planting probably represented a single population whilst the two sets of equivalent C/D/E isolates were distinct both from each other and from the A/B population. This is consistent with A/B cankers arising largely from infections brought in with the trees and the C/D/E cankers arising mainly from *de novo* infections in the orchards after planting. Further, it suggests that the isolates spreading into the NIHPBS represented a population differentiated from that moving into the other two sites. Too few C/D/E canker isolates from ADAS were available to ask whether the 'West Midlands' population distinct from the 'Kentish' one but all the type 20 isolates came from Rosemaund (representing four of the six C/D/E isolates typed) suggesting that this may well be the case. Whilst the A/B isolate population as represented in the trees in this

experiment is distinct from both the C/D/E populations, geographical differentiation between the two (or even three) C/D/E populations suggests that the A/B isolates represent an amalgam of the populations at the three contributing nurseries, biased according to the number of cankers each nursery 'contributed'.

Table 3. Comparison of the frequencies of occurrence of molecular types for four populations of isolates. Figures represent number of times molecular types occur (*e.g.* for A/B cankers from NIHPBS, 19 molecular types appeared only once and six appeared twice).

Frequency of	NIHPBS		HRI/ADAS	
Occurrence	A/B	C/D/E	A/B	C/D/E
1	19	11	6	17
2	6	5	2	4
3 or more	*3*	*1*	*2*	*3*
Ratio of 1:2	**3.2:1**	**2.2:1**	**3.0:1**	**4.25:1**

OBJECTIVE 5C:
Identification of stages of tree production in the nursery most susceptible to infection by *N galligena*

Preparation of inoculum: An apple cv Bramley was inoculated with an isolate of *N galligena* of known DNA fingerprint (isolate W3 ex Wye College). After 10 days rotted tissue was isolated onto Sugar Nutrient agar and yeast and incubated at 20^{0}C to allow growth and sporulation, which occurred after 4-6 weeks. Conidia were scraped from the plates into sterile distilled water, filtered through muslin and the conidial concentration estimated and adjusted to 4 x 10^4 spores per ml using a haemocytometer slide. This inoculum was either used directly (high spore concentration) or diluted to 4 x 10^3 (medium spore concentration) or diluted to 4 x 10^2 (low spore concentration). This inoculum concentration was used for all inoculations except at budding when an increased concentration of 4.5 x 10^5 was used. For each inoculation 30 microlitres of inoculum were used, which was equivalent to approximately 1200 spores for the high concentration, 120 spores for the medium concentration and 12 spores for the low concentration.

Tree propagation and inoculation: All stages of propagation were carried out at HRI-East Malling. Trees were inoculated at three main stages of propagation; removal of side shoots of rootstock (defeathering), budding or heading back. In July 1995 lined out M9 rootstocks were defeathered up to a height of 20cm and 30 microlitres of inoculum applied to 3 wounds per tree on 50 trees for high and low spore concentrations and 48 trees for the medium. In August 1995 Queen Cox budwood was collected and 30 microlitres of inoculum applied to the budwood before budding. Again 50 trees were treated with the high or low spore concentration and 49 with the medium spore concentration. In March 1996 the rootstocks were headed back initially to with in 15cm of the bud and one week later removed entirely. The cut surface was inoculated with 30 microlitres of inoculum, 49 trees with the high and 50 trees with the medium or low concentration. Any remaining shoots were pruned in June 1996 leaving one main Queen Cox scion shoot. At each stage of propagation 48 control trees were included which were inoculated with sterile distilled water.

In December 1996 all remaining healthy trees were removed from the nursery site and held in cold store. The following March these trees were planted out in an orchard site at HRI East Malling.

Assessment: Trees were monitored regularly both in the nursery and orchard site for any signs of canker. The site of any obvious cankers was recorded and the cankers collected and stored at –20^{0}C. In August 1996 the height of each tree, measured from the graft union, was recorded and the trees graded.

Isolation of *Nectria galligena* and DNA fingerprinting: Cankers were isolated onto PDA amended with rifamycin and iprodione both at 20ppm. Isolates of *Nectria galligena* obtained were grown on cellophane on PDA (Rif/ipro), the mycelia harvested and DNA extracted using the method of Lecellier and Silar (1994). Extracted DNA was subjected to PCR-RFLP analysis of the intergenic spacer region (IGS), following the technique developed under objective 1. The PCR products were digested separately with restriction enzymes; *Hinf*I, *Hae*III, HhaI and *Taq*I Banding patterns (DNA fingerprints) for the *N galligena* isolates were compared to those of the original isolate W3 on a 2% metaphor agarose gel.

Results

Most cankers developed within two years of inoculation, some took only three months to develop and others three years. The frequency of canker development correlated with the spore concentration of the inoculum, which was most obvious in trees inoculated at budding time (Table 4). The lowest concentration used, equivalent to about 12 spores per inoculation site probably most closely resembled the natural situation. The majority of trees inoculated at defeathering or at heading back developed canker even at the lowest concentration. Fewer trees inoculated at budding-time developed canker, particularly at the lower concentration. All of the trees inoculated at defeathering with the highest spore concentration developed canker in the nursery. Even at the lower spore concentrations the majority of tree inoculated at this stage developed canker in the nursery, indeed only 6% of the trees inoculated at defeathering survived for planting out in the orchard. Half the trees inoculated, with the heaviest spore concentration, at budding and 60% of those inoculated at heading back also developed canker in the nursery.

Table 4. Numbers of trees developing canker following inoculation with *Nectria galligena*

*	Conc. of conidia	No. trees inoculate	Number of trees developing canker (cumulative totals)										% trees with canker
			Nov 95	Jan 96	Jun 96	Aug 96	Dec 96	Jan 97	Mar 97	Aug 97	Aug 98	Oct 98	
DF	Low	50	0	0	34	38	43	43	43	43	43	43	**86**
	Med.	48	0	1	39	40	47	47	47	48	48	48	**100**
	High	50	1	3	48	48	50	50	50	50	50	50	**100**
Bud	Low	50	1	1	1	4	5	5	5	5	7	16	**32**
	Med.	49	1	2	6	9	9	9	9	9	13	14	**29**
	High	50	7	10	17	25	25	25	25	30	36	39	**78**
HB	Low	49	0	1	2	3	5	6	10	31	31	39	**78**
	Med.	50	0	0	10	11	17	18	27	44	46	49	**98**
	High	50	0	0	21	21	30	34	34	48	50	50	**100**
Con	Water	48	0	0	0	0	0	0	0	1	6	8	**17**
	Totals	**494**	**10**	**18**	**181**	**199**	**231**	**237**	**250**	**309**	**330**	**356**	

* **Inoculation time and location (DF = Defeathering in June 1995: Bud = Budding in August 1995: HB= Heading back in March 1996: Con = Control, water only)**

Trees inoculated at budding or heading-back also developed cankers in orchard site (Table 5). No cankers developed on control trees in the nursery, but six trees developed canker in the orchard. All cankers collected in 1996 (in the nursery) were found either on the rootstock or around the graft union, which were associated with the original inoculation points. Of cankers collected in 1997 (orchard) 13% were found at locations other than the inoculation sites, including on side branches relatively high up the tree. In 1998 59% of cankers were from locations other than the point of inoculation, but most were on low branches located near the graft union.

Table 5. Location of trees when cankers developed

Inoculation time and location	Spore concentration of N galligena	Trees with canker (%)			
		Nursery (1995-96)	Orchard 1997	Orchard 1998	Orchard 1999
Defeathering June 95	Low	86	0	0	0
	Medium	98	2	0	0
	High	100	0	0	0
Budding August 95	Low	10	0	4	18
	Medium	18	0	8	4
	High	50	10	12	6
Heading back March-96	Low	10	53	0	16
	Medium	34	54	4	6
	High	60	36	4	2
Controls	None Water only	0	2	10	4

Trees inoculated at budding grew better in the nursery and were of better quality compared to trees inoculated at other stages (Table 6). *Nectria galligena* was successfully isolated from 47 cankers, 33 of which were from heading-back treatment, 8 from budding treatment and 6 from defeathering treatment from different years of collection up to 1998, representing about 13% of the total cankers that developed. No cankers were analysed from collections in 1999. All isolates of *N. galligena* were found to be identical to the original inoculum (isolate W3). As statistical analysis suggests that as all of the 13% tested were identical to the original isolate then it is probable that the remaining isolates would also be identical. No *N. galligena* was isolated from any of the cankers found on the control trees.

Table 6. Tree height and grade August 1996

Inoculation time and location	Spore concentration of *N galligena*	Mean tree height from graft union cm	% grade one trees
Defeathering June 95	Low	69.2	16
	Medium	64.5	6
	High	41.4	0
Budding August 95	Low	91.6	62
	Medium	91.0	72
	High	90.8	50
Heading back March-96	Low	78.5	34
	Medium	80.5	26
	High	79.6	34
Controls	None Water only	89.9	66

The trees in orchard two (Apple Tree Corner) consisted of cvs Cox and Worcester. This orchard was also less than five years old and both apple cultivars were from a nursery in Kent. The orchard was divided into three areas. Area I was adjacent to an old (30+ years) apple orchard of cvs Cox and Spartan of different nursery origin. Area III was furthest from the old apple orchard and area II between the two. Cankers were collected from areas I, II and III during spring 1997, from young and old wood and the tree location, cultivar and wood age recorded as above. A random sample of cankers was also collected from the old orchard adjacent to area I. All cankers were stored at -20^0C prior to isolation.

The orchard in Herefordshire was located at Pencoyd and consisted of trees of cvs Elstar, Gala and Jonagold, planted in 1989 or 1992, the two plantings differing in nursery of origin. This orchard was similarly sampled in spring / summer 1997 and the cankers stored at -20^0C until needed.

Isolation of *Nectria galligena* and DNA analysis: *Nectria galligena* was isolated from cankers on to PDA amended with rifamycin and iprodione both at 20ppm. Isolates of *N. galligena* were grown on cellophane on PDA (rif/ipro), the mycelia harvested and DNA extracted using the method of Lecellier and Silar (1994). Extracted DNA was subjected to PCR-RFLP analysis of the intergenic spacer region (IGS), following the technique developed under objective 1. The PCR products were digested separately with restriction enzymes; *Hinf*I, *Hae*III, *Hha*I and *Taq*I. The *N galligena* isolates were then assigned groups based on the banding patterns obtained on a 2% metaphor agarose gel.

Statistical analysis: Comparisons were made of the banding patterns obtained from the *N galligena* isolates from the different orchard areas in each young orchard/old orchard combination and Chi^2 analysis carried out.

Results

Hop Garden: Ninety-two isolates of *N galligena* were obtained from a total of 135 cankers from Cox and Gala representing areas A and C of the orchard. 62 cankers were collected from the old pear orchard and 59 cankers from the adjacent old apple orchard, from which 42 and 12 isolates of *N galligena* were obtained respectively. 157 cankers were collected from area B but these were not isolated from. The success rate of isolation of *N galligena* from cankers ranged from 20-68%. DNA analysis resulted in 16 overall groupings, 5 of which were commonly observed, the other groups occurring infrequently. Comparisons were made of the patterns obtained from *N galligena* isolates from wood from areas A (51 isolates), C (41 isolates) and the old pear orchard (42 isolates). Eighteen groups were found (P= 0.45). Similarly comparisons were made between isolates from Gala (51 isolates), Cox (41 isolates) and pear (42 isolates). Again 18 groups were identified (P=0.19). It was therefore concluded that there were no significant differences between *Nectria* isolates obtained from either Cox or Gala or from any of the two areas of the young orchard and the old pear orchard.

Apple Tree Corner: One hundred and one isolates of *N galligena* were obtained from a total of 162 cankers from Cox and Worcester from areas I, II and III of the young orchard, representing a 62% success rate. A further 18 isolates were obtained from 74 cankers collected from the adjacent old apple orchard. Chi^2 analysis of the *N galligena* isolates from areas I (27 isolates), II (34 isolates), III (27 isolates) and the old apple orchard (10 isolates) resulted in 27 groups, although a number of these only occurred once or twice (P=0.072). There was no significant difference between isolates from areas I or II or the old apple orchard. However there was a significant difference between *Nectria* isolated from area C compared to *Nectria* isolated from the rest of the orchard and the old orchard. Area III contained a isolates of a single type of *Nectria* which did not occur in the rest of the orchard but did occur in Hop Garden orchard and the adjacent old pear orchard. There was no significant difference (P= 0.3604) between *Nectria* isolates from Cox (76 isolates) and Worcester (12 isolates), but there were relatively few isolates from Worcester.

Pencoyd: Sixty seven isolates of *N galligena* were obtained from 157 cankers collected from Elstar, Gala and Jonagold (1989 planting), representing a 42% success rate. DNA analysis was conducted on 61 isolates representing 14 different areas in the orchard (cultivar, year, site combinations), from which 28 different groups were identified. There was therefore insufficient numbers of isolates of *N galligena* to perform a Chi^2 analysis.

Discussion
In two of the young orchard/old orchard site combinations investigated there was no difference in the populations of *N galligena* between orchard area, cultivar or young or old orchard. This suggests that there is movement of *Nectria* inoculum between and within orchards and supports the traditional accepted view of epidemiology of *N galligena*. However, this interpretation is based on the assumption that the *Nectria* populations from the French nursery that supplied the Gala in Hop Garden orchard and the nurseries that supplied the trees in the old apple and pear orchards adjacent to Hop Garden and Apple Tree Corner are distinct from those in the Kent nursery supplying the other trees in the young orchards. It was not possible to test this assumption directly by obtaining cankers from the various nurseries. However additional data could support the movement of *Nectria* spores between orchards. Area III of Apple Tree Corner (ATC) contained *Nectria* isolates that were not present in Areas I and II or in the adjacent old orchard. This isolate was present in the old pear orchard adjacent to Hop Garden orchard and in areas A and C of that orchard. Area III of ATC is separated from the old pear orchard by a wood, which effectively screens it from the pear orchard. However two to three years previously the wood was cut down which exposed the young apple trees in area III of ATC to the possibility of *Nectria* inoculum from the pears. This offers one explanation for the presence of the *Nectria* isolate in the area III of ATC, which would also support the idea of movement of *Nectria* spores from one orchard to another.

Conclusions
- DNA analysis of *Nectria* isolates obtained from different cultivars, different areas within orchards and from adjacent orchards for two sites in Kent showed that there was no significant between the population of *Nectria*.
- This therefore suggests that there is movement of *Nectria* inoculum between the orchards and within the orchards, but further data must be obtained to allow conclusions to be drawn on the possible origins of *Nectria* infection and methods of dissemination.

Bramley's Seedling Trial, NIHPBS, Loughgall
In what was a similar type of trial to the Aylsham trial (Described in the previous report) two small orchards of Bramley's Seedling trees were planted at NIHPBS, Northern Ireland. Phase I was planted in 1997 and comprised 75 trees. Twenty-five of the trees were Bramley Seedling on M26 rootstock obtained from Nursery A (Blackmore), 25 were on rootstock MM106 from Nursery B (Leech) and 25 were on rootstock M9 from nursery C (Brinkman). The trees were planted as fully randomised single trees.

At the assessment carried out in spring 2001 all of the trees in the trial had at least one canker. Significant (P=0.05) differences were observed in the number of cankers developing on trees from each of the nurseries (Fig 1).

However, because the trees were on different rootstocks and in the absence of differentiating molecular data no clear conclusions can be drawn from this experiment.

Phase II of the Bramley's Seedling orchard was planted in 1998. Seventy-two trees, all on rootstock M9 were planted in single tree plots. Twenty-four trees had been obtained from Nursery A (Blackmore) and 24 each from Nursery C (Brinkman) and Nursery D (Matthews). At the assessment carried out in spring 2001

significantly (P=0.05) more trees from Nursery C had canker, compared to the other two nurseries. Furthermore these trees had significantly (P=0.05) more cankers present (Fig 2 & 3).

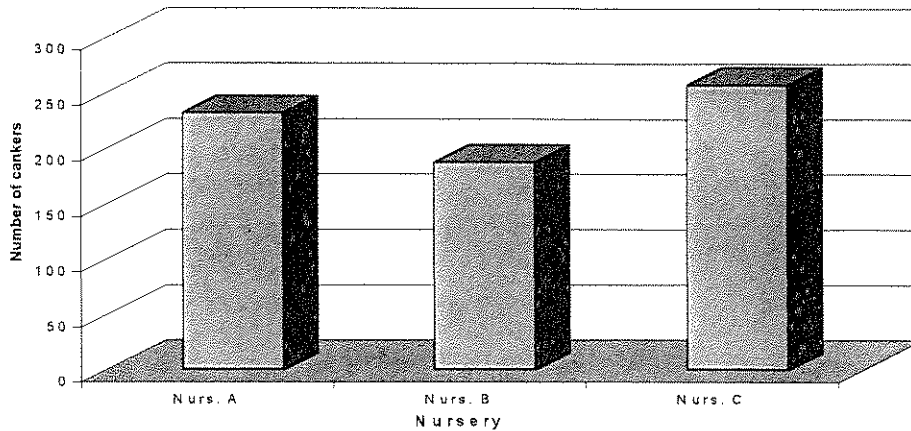

Fig 1. Number of cankers developing on Bramley Trial Phase 1

This may suggest that the nursery is a source of inoculum. No molecular analysis has yet been carried out which might relate particular isolates to particular nurseries. In addition there may be inherent characteristics in certain batches of trees, e.g. vigour which may predispose them to expression of infection.

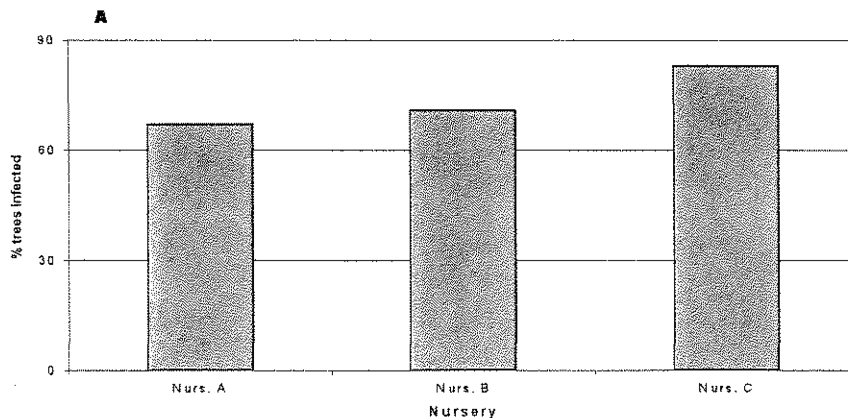

Fig 2. Percentage trees infected (A) in Bramley's Seedling Trial Phase 2

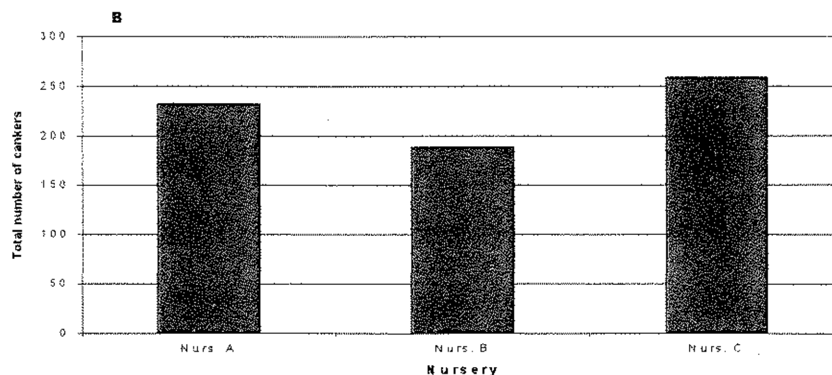

Fig 3. Percentage trees infected (A) in Bramley's Seedling Trial Phase 2

Discussion

Fewer cankers developed from inoculations at budding than at the other timings and these trees also grew better suggesting they were relatively disease-free. This might have been because the inoculations made at budding time were less successful. Inoculum at the other timings was applied to fresh cut surfaces, which is known to result in the spores being sucked into the tissue, whereas inoculum at budding time was applied to the budwood prior to budding and not the cut surface. Therefore possibly fewer or no spores entered the tissue. Alternatively at this stage, the host might be more successful in temporarily sealing up the *Nectria* spores thus not allowing the canker to be expressed, but events might subsequently favour *Nectria* development resulting in canker expression at some later date. In this case more canker might be expected to develop in the orchard, which was the case, but at a low rate each year, such that by 1999 canker had developed on 22% of trees inoculated at budding time with the low inoculum rate. This low inoculum rate, approximately equal to about 12 spores, is more equivalent to natural Nectria infection. In newly planted commercial orchards, tree losses per season, which could be attributed to nursery canker, can be 10% or more. *Nectria* inoculations at the other infection sites developed into canker more rapidly and most trees had developed canker within 12-18 months from inoculation and mostly in the nursery.

Most cankers initially arose at the point of inoculation, but cankers appearing in 1998 were mostly located either on the lowest side shoots near the graft union or in the branch angle of this side shoot. These cankers may have developed from internal spread from the original inoculation point or through aerial spread.

Conclusions

- Canker develops more rapidly from inoculations made at the time of defeathering or at heading back compared to those made at budding time. Which results in canker expression up to three or four years after the inoculation.
- It is difficult to decide from the data whether the differences in speed of canker development are related to differences in the success of the inoculations or to differences in the ability of the tree to temporarily seal off the Nectria fungus, related to the inoculation site.

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Publications and Technology Transfer

Langrell, S. R. H. & Barbara D. J. (2001). Magnetic capture hybridisation for improved PCR detection of *Nectria galligena* from lignified apple extracts. *Plant Molecular Biology Reporter* **19**, 5 – 11.

Langrell, S. R. H., Swinburne, T. R. & Barbara D. J. Comparisons of PCR methods for assessing intra-specific variation in Nectria canker fungus (*Nectria galligena* Bres.). *European Journal of Plant Pathology.* (Submitted).

Papers are currently in preparation for submission to Scientific Journals. These will particularly cover the results of the field experiments.

A number of talks were given by Dr. Angela Berrie to apple growers in southern England, by Dr. Alistair McCracken in Northern Ireland and by Dr. Tom Locke about apple canker control.

Growers also had the opportunity to visit the 'Millennium Orchards' at HRI, East Malling and NIHPBS, Loughgall.

A number of popular articles were written by Dr. Berrie or Dr. McCracken to keep growers informed about the progress of the apple canker project (examples appended)

Talks and orchard visits were widely reported in the local press.

Possible Future Work

The results of the previous project have clearly indicated that there is a genuine risk of infection occurring at the propagation stage in nurseries, particularly at the budding stage. It is therefore imperative to investigate the epidemiology of the pathogen at this stage. This would include:

At what stage of propagation can infection occur?
Budding?
Heading back?
De-feathering?
Natural leaf-fall?

Can plants be treated at any of these stages to reduce canker risk?

How do planting conditions e.g. wet soils predispose canker development?

Such work could be successfully carried out at HRI East Malling with orchards being planted at a range of sites in England and Northern Ireland

How do the more recent fungicides control canker as spring /autumn sprays?

Fungicides with canker control activity could be tested on both artificially inoculated and artificially inoculated trees in N. Ireland

In addition to these practical / applied issues there would be significant benefit in developing a system of detection / differentiation of *N. galligena* in wood. The probability of successfully developing such a system using micro-satellites is high when used on wood displaying symptoms. The development of a reliable and robust method to detect *N. galligena* in symptomless wood is less well assured but should nonetheless be pursued.

It is envisaged that the applied and molecular aspects of the project could be developed in parallel

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