Horticultural Fellowship Awards

Interim Report Form

DRAFT REPORT FORM JDK 02/04/12 © 2012 Agriculture and Horticulture Development Board

Project title:	Succession planning to sustain the UK's expertise in field and laboratory plant pathology research and development
Project number:	CP 90
Project leader:	Dr Angela Berrie East Malling Research
Report:	Annual report, November, 2014
Previous report:	Annual report, October, 2013
Fellowship staff: ("Trainees")	Dr Robert Saville
Location of project:	East Malling Research
Industry Representative:	Andrew Tinsley, Horticultural Development Company
Date project commenced:	7th November 2011
Date project completed (or expected completion date):	6th November 2016

 © 2012 Agriculture and Horticulture Development Board

DISCLAIMER

AHDB, operating through its HDC division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

Copyright, Agriculture and Horticulture Development Board 2016. All rights reserved.

No part of this publication may be reproduced in any material form (including by photocopy or storage in any medium by electronic means) or any copy or adaptation stored, published or distributed (by physical, electronic or other means) without the prior permission in writing of the Agriculture and Horticulture Development Board, other than by reproduction in an unmodified form for the sole purpose of use as an information resource when the Agriculture and Horticulture Development Board or HDC is clearly acknowledged as the source, or in accordance with the provisions of the Copyright, Designs and Patents Act 1988. All rights reserved.

AHDB (logo) is a registered trademark of the Agriculture and Horticulture Development Board.
HDC is a registered trademark of the Agriculture and Horticulture Development Board, for use by its HDC division.
All other trademarks, logos and brand names contained in this publication are the trademarks of their respective holders. No rights are granted without the prior written permission of the relevant owners.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name]
[Position]
[Organisation]

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]
[Position]
[Organisation]

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

 © 2012 Agriculture and Horticulture Development Board

Grower Summary

The nature of the fellowship projects means that a grower summary is not appropriate at this stage.

Science Section

Objectives

As part of the training fellowship three projects were initially proposed to encompass some of the training requirements vital for field and laboratory plant pathology research and development. The fellowship projects were reviewed at the beginning of 2014 to incorporate new objectives. The fellowship projects for the third year of the fellowship are as follows;

(1) Continuation of the apple rot survey and determination of the causative agents of apple rots to contribute towards the sustainable control of storage rots of apple.
(2) Utilise a new metagenomic assay in development at EMR to determine the endophytic profile within commercial strawberry plants for future research on the role of endophytes on plant tolerance/resistance to pests/diseases in relation to host genotypes and external conditions.

Project 1: Sustainable control of storage rots of apple

Introduction

Fungal rots can result in significant losses in stored apples, particularly in fruit stored beyond January. Certain pack houses will record losses due to rots for individual bins of fruit, thus relating the loss to particular orchards, harvest time and pre-harvest factors, however they rarely identify the rots present. It is important to identify the rot profile in stored apples over time to build a dataset (including orchards, harvest time and pre-harvest factors) from which to base management strategies. In previous surveys Nectria, Botrytis, brown rot (Monilinia), Penicillium, Phytophthora and Gloeosporium have been identified as the main rots in apple. Other rots such as those caused by *Colletotrichum sp., Fusarium sp., Botryosphaeria sp.* and *Phomopsis sp.* have been increasing in incidence. A greater understanding of the epidemiology and orchard factors contributing to rot development has helped in informing management strategies to reduce their prevalence.

 © 2012 Agriculture and Horticulture Development Board

The concept of rot risk assessment was introduced via the Apple Best Practice Guide (Webster *et al*. 2001). The rot risk assessment takes account of various pre-harvest factors to predict the level of rot likely to occur in store and thus inform a management strategy, be it pre-harvest treatments, selective picking or storage term, to minimise losses in store. The factors assessed pre-harvest are; daily rainfall, orchard factors, fungal inoculum (particularly brown rot and canker), crop load, % bare ground (Phytophthora), % crop <0.5 metre from the ground, orchard rot history and fruit storage potential (mineral composition and firmness). For example, Phytophthora rot risk is influenced by three key factors; Rainfall in the 15 days prior to harvest, % bare ground and % crop <0.5 metre from the ground (Table 1).

Table 1. Factors influencing the risk of Phytophthora rot (from Apple Best Practice Guide, Webster et al. 2001)

Factor	Criteria for risk
(1) Rainfall in 15 days prior to harvest	low or no rain = low risk 20 mm or >= high risk
(2) % bare ground	100% bare ground (overall herbicide) = high risk 0% bare ground (overall grass or mulch or weed cover) = low risk
(3) % crop <0.5 metre from the ground	15% or >= risk

In addition to rot risk assessment other management strategies can be employed to minimise losses in store such as selective picking whereby only undamaged fruit is harvested and all fruit below 0.5 metres above the ground is excluded. This reduces the risk of introducing fungal rots, such as brown rot and Penicillium rot which establish on damaged fruit, and also Phytophthora rot which is prevalent on low hanging fruit, into the bin.

Pre-harvest fungicides applied for rot control are generally applied 2-4 weeks before harvest resulting in a high risk of residues in the fruit. By applying the recommendations set out in the rot risk assessment as part of an IPM approach, such treatments could be avoided, thus reducing the risk of pesticide residues on fruit whilst reducing the financial and environmental costs of pesticide application.

Data available from rot surveys undertaken over the last 80 years reveal interesting trends in the rot profile overtime (Figure 1) which reflects changes in apple growing practices.

 © 2012 Agriculture and Horticulture Development Board

Fungicide use (chemistry available and application timing) have changed markedly from post-harvest drenching in the past to flowering and pre-harvest application currently.

Advances in storage technologies has led to significant reductions in losses and also influenced the rot profiles observed. Barn stored fruit in the 1930's in which average losses in Cox of over 25% were recorded and the dominant rots were caused by *Neofabraea sp*. and *Botrytis* whilst modern day refrigerated controlled atmosphere storage with ethylene management technologies to control ripening have average losses in Cox of less than 3% and the dominant rots are caused by *Monilinia fructigena* and *Neonectria ditissima*. In addition, changes in climatic conditions and orchard practice will influence the rot profile over time.

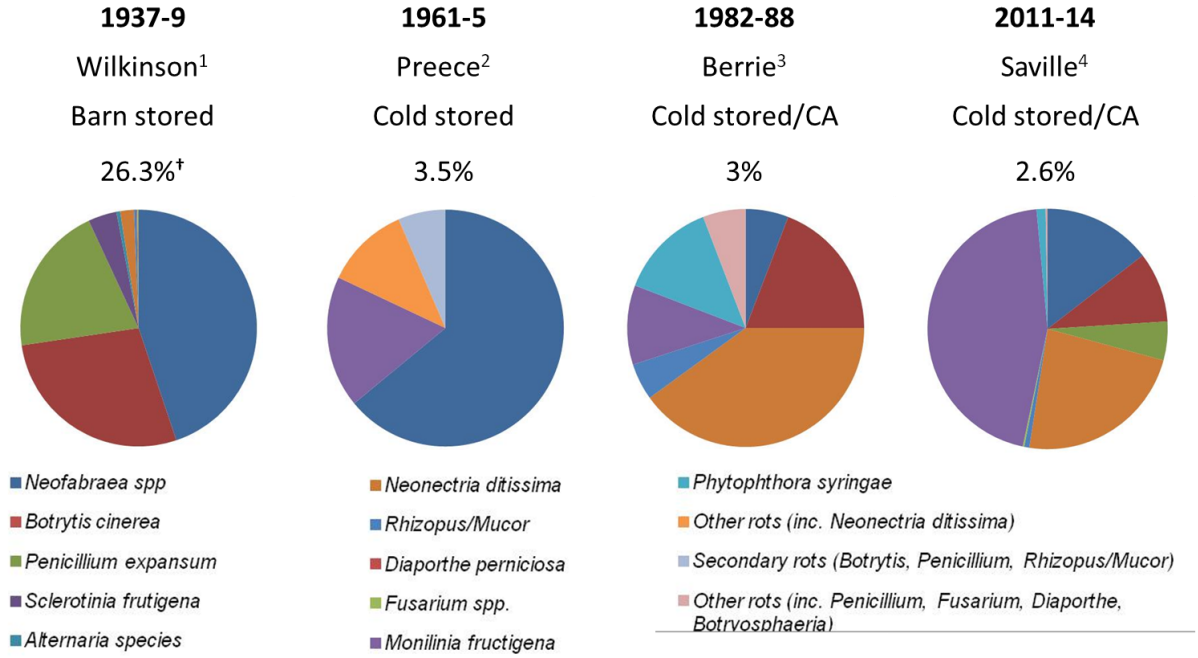

Figure 1. Data compiled from four rot surveys spanning the last 75 years.

The data set is for Cox as this is the common cultivar recorded across all surveys.

[1]Wilkinson, 1984, [2]Preece, 1967, [3]Berrie, 1989, [4]Saville, 2013,

† Average total losses due to rots during the survey period.

The categorisation of taxa in the legend are described as recorded in the literature so some inconsistencies between data sets are present i.e. *Sclerotinia frutigena* is a synonym of *Monillinia fructigina* and rots have been grouped in certain surveys e.g. 'other rots'. As far as possible common colour coding has been used to represent these inconsistencies.

It is important to continue the rot survey to monitor changes in rot profiles over time and, in turn, inform and prioritise management strategies accordingly. The results from the 2013/14 rot survey will be presented here in the context of previous rot surveys. Using molecular

 © 2012 Agriculture and Horticulture Development Board

identification techniques a collection of *Neofabraea* sp. collected as part of the rot survey has been characterised to the species level, further increasing our knowledge of the rot-causing pathogens and further contributing to strategies to mitigate loss.

Methods

Survey

Five pack houses were visited in Kent between January and March 2014 (Table 2). Rots were assessed on the grader of whatever variety was being graded at the time of the visit. Rots were identified visually and numbers recorded. Unidentified rots were cultured on to potato dextrose agar and identified from spores or characteristic culture growth.

Table 2. Fruit pack houses visited between January and March 2014

Pack house	Location	Number of times visited
Newmafruit Farms Ltd	Howfield Farm, Chartham Hatch, Kent	6
F W Mansfield & Sons Ltd	Nickle Farm, Chartham, Kent	6
The Breach	Goudhurst, Kent	4
Bardsley & Sons	River Farm, Staplehurst, Kent	4
J L Baxter & Son Ltd	Amsbury Farm, Hunton, Kent	4

Molecular identification of the **Neofabraea** *spp. complex*

A collection of *Neofabraea* spp. isolates (n = 104) collected over two storage seasons mostly from pack houses which store fruit grown in Kent but also including samples from a pack house in Herefordshire. Spore morphology and molecular identification (on a subset of 70 isolates) were used to determine the species level of the isolates. Spore production was promoted by sub culturing the isolate collection on to different media that have been reported in the literature to promote sporulation. On those that sporulated (very few did due to age of the cultures) spores were harvested and visualised using a microscope.

Species were determined based on the spore morphology descriptors in Sutton *et al.* (2014). The spore morphology of the *Neofabraea* spp. are overlapping with as much intra-species

 © 2012 Agriculture and Horticulture Development Board

variation as inter-species variation, therefore, to complement the identifications based on spore morphology, molecular identification was also used. DNA was extracted from a subset of 70 isolates in the collection. The DNA was amplified from two phylogenetically informative regions (ITS and B-tubulin) and sequenced. The sequence data was then queried against reference sequences of the *Neofabraea* spp.

Results and Discussion

2013/14 survey

A total of 53 samples of fruit were surveyed during the 24 visits spanning from mid-January to mid-March. In total nine different cultivars were surveyed: Braeburn (11), Cox (12) and Gala (10) were the most common cultivars surveyed. The majority of samples surveyed were picked in September (17) and October (23), due to the late harvest of 2013 surveyed samples were also picked as late as November (9) and December (1). A summary of the rot survey data is presented in Table 3.

Table 3. Summary table of rot survey data collected during the 2013/14 storage season. The table shows the average percentage loss attributed to each rot for each cultivar recorded during the survey together with the number of samples recorded for each cultivar and the average percentage loss.

Cultivar	Average % of loss attributed to each rot;														Number of samples	Loss (%)
	Brown rot	*Botyrtis*	*Phytopthora*	*Penicillium*	*Nectria*	*Gloeosporium*	*Fusarium*	*Mucor*	*Botryosphiria*	*Phomopsis*	Stalk	Eye	Cheek	Core		
Braeburn	32.2	4.5	25.7	3.1	10.4	0.3	0.0	24.2	0.0	0.0	0.0	0.0	0.0	0.0	11.0	1.3
Bramley	50.3	6.6	0.0	4.6	27.7	0.0	10.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.9
Cameo	0.0	13.5	0.0	2.2	63.3	0.0	20.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	1.0
Cox	46.7	12.1	3.6	2.7	30.8	3.7	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	12.0	1.1
Egremont Russet	15.7	1.0	0.0	3.7	75.0	4.2	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	1.0
Gala	34.8	7.7	4.3	0.3	51.2	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	10.0	0.9
Jazz	0.0	42.9	14.3	4.8	8.3	0.0	0.0	29.8	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.6
Other dessert	2.3	42.9	1.8	1.2	49.4	0.6	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	4.0	1.2
Overall average	**22.7**	**16.4**	**6.2**	**2.8**	**39.5**	**1.1**	**3.9**	**7.1**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	-	**2.6**

 © 2012 Agriculture and Horticulture Development Board

As with the other surveys in recent years Nectria rot (*Neonectria ditissima*) has surpassed brown rot (*Monilinia fructigena*) as the overall dominant rot. This reflects the high levels of inoculum present (in the form of cankers) in the orchards which have increased in recent years because of the succession of canker favourable seasons. Due to the late harvest, coinciding with high rainfall the Phytophthora rot (*Phytophthora syringae*) risk was high.

The risk was predicted to be high according to the rot risk assessment which takes into account the rainfall in the 15 days prior to harvest (>20mm = high risk, Table 4, red highlighted text). However, despite the risk, Phytophthora rot incidence was comparable to recent relatively dry years (Table 4, green highlighted text), suggesting that the rot risk guidelines are being observed. The largest average % of loss attributed to Phytophthora rot was observed for cultivar Braeburn, consistent with the fact that this cultivar is harvested particularly late and as such was exposed to higher rainfall in the 15 days prior to harvest.

Soil is an important source of infection for Phytophthora (and Mucor/Rhizopus) and with the late, wet harvest many of the bins were contaminated with soil. Phytophthora and Mucor/Rhizopus were particularly high in incidence in contaminated bins (Figure 2). Gloeosporium rot (*Neofabraea* sp.) occurred at low incidence (16% of apple samples surveyed contained at least one Gloeosporium rot) and were only recorded in particularly susceptible cultivars (Cox and Egremont Russet).

This result is consistent with a recent trend in the reduced incidence of this rot over the last 2 surveyed growing seasons which followed three successive years of increasing incidence (Figure 3). Mouldy core (*Fusarium tricinctum*) is particularly evident in the open calyx varieties (Bramley and Cameo) as has been observed in pervious projects on this disease.

Table 4. Phytophthora risk table for the 2013 growing season and 5 years previously for comparison. Also included for comparison is 1995, a particularly wet harvest year. Hypothetical harvest dates are highlighted in blue, harvest dates associated with a risk (i.e. > 20 mm of rain fell in the 15 days prior to harvest) are highlighted in red. The percentage of apple samples in which Phytophthora was present in the total surveyed samples is highlighted in green.

 © 2012 Agriculture and Horticulture Development Board

Harvest date	Rain (EMR) in 15 days pre-harvest > 20mm = risk						
	1995	2008	2009	2010	2011	2012	2013
20 August	0	-	-	-	9.8	21.6	6.6
25 August	0.1	-	-	-	22	22	50.8
1 September	2.9	15.6	3.6	59.4	29	9.6	49.8
5 September	13.8	29.8	10.4	59.4	35.2	9.6	45.4
10 September	35.8	42	9.8	22	23.4	4.8	9.8
15 September	66.2	42.6	25.6	28.8	20	5	29.6
20 September	109.7	15.8	16.4	29.2	18	5	34.8
25 September	99	4.4	17	9	8.8	53	31.2
30 September	89.7	8.2	0.8	23.6	8.2	56.2	13
5 October	43.4	25.2	8.4	65.6	6.4	96	19.4
10 October	32.7	28	37.8	63.6	8.2	73.8	22.8
15 October	6.9	25.4	38.2	47.4	7.6	72.6	82.8
20 October	0.8	18.6	34.8	10.6	9.2	60.4	99.2
31 October	3.3	28.8	9.2	24.4	26.2	56	73.4
5 November	*	*	*	*	*	*	90.8
10 November	*	*	*	*	*	*	86.6
15 November	*	*	*	*	*	*	66.2
20 November	*	*	*	*	*	*	33.2
25 November	*	*	*	*	*	*	21.6
30 November	*	*	*	*	*	*	13.8
5 December	*	*	*	*	*	*	7.8
% apple samples with Phytophthora	?	40.7	18.6	66.7	35.7	32	26

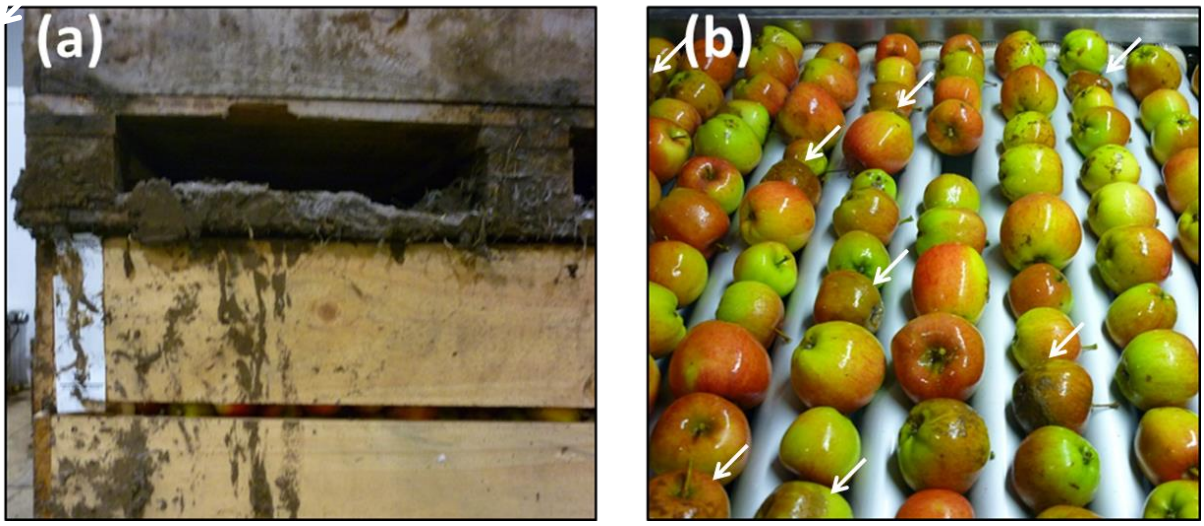

Figure 2. A Braeburn crop harvested on 04/11/13 and surveyed on 21/01/14

(a) the muddy bins that fruit was harvested and stored in

(b) the same consignment of fruit on the grading line.

10% losses were recorded for this consignment (evident rots highlighted with white arrows) the majority of losses resulting from Phytophthora and Mucor/Rhizopus.

 © 2012 Agriculture and Horticulture Development Board

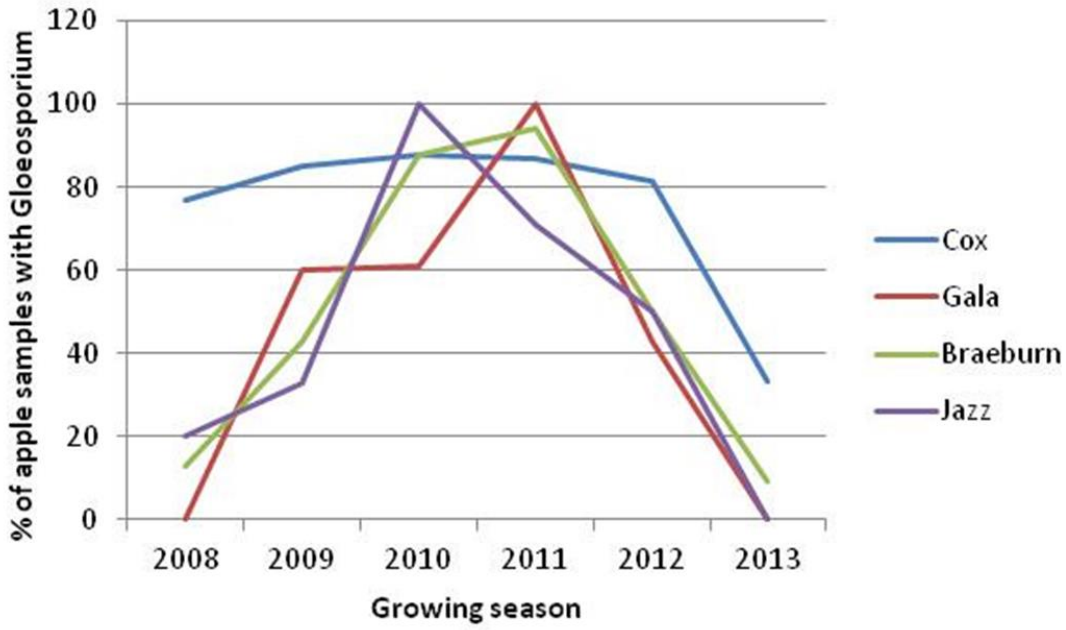

Figure 3. Neofabraea incidence of four susceptible cultivars in rot surveys spanning 2008-2013 growing seasons the graph shows the percentage of apple samples with Neofabraea (Gloeosporium).

Molecular identification of the Neofabraea *spp. complex*

On the basis of increasing incidence of Gloeosporium rot (Neofabraea) occurring in the rot survey in the years preceding 2011 (Figure 3) a collection of isolates was curated over the next two storage seasons and were characterised to the species level in early 2014. Of the 104 isolates, it was not possible to identify 11 of the isolates on any of the criteria used (data not shown). Unfortunately, spores were only generated in a small proportion of isolates (20%). Where spore morphology and molecular identification data was available, both were in agreement. Of the 70 isolates for which molecular identification was undertaken; 30% did not amplify, 46% were identified as *N. alba*, 13% were identified as *N. perennans*, 9% were identified as *N. perennans* or *N. malicorticis* (only ITS sequence data was available for these isolates which is insufficient to distinguish *N. perennans* and *N. malicorticis*). A single isolate, R142/12/5, did not match any of the reference sequences used. Further analysis revealed that this isolate is the putative new *Neofabraea* spp. initially described by de Jong *et al*. (2001) and subsequently named *Cryptosporiopsis kienholzii* (Spotts *et al*., 2009). To the best of our knowledge, this is the first reported recording of this species in the UK.

 © 2012 Agriculture and Horticulture Development Board

The metadata associated with each of the isolates (i.e. cultivar and location collected) means that a picture of the species distribution can be drawn (Figure 4). In terms of cultivars (Figure 4a), *N. Alba* is dominant in all cultivars apart from Cox in which *N. perennans* is the dominant species. In terms of the geographic distribution of species (Figure 4b), in this small study, isolates were only collected from Kent and Herefordshire, the two major apple growing regions in the UK. Despite the small scale of this study, obvious trends are evident in the species distribution with *N. alba* dominant in Kent whilst *N. perennans* is the dominant species in Herefordshire. The dominance of *N. alba* causing Gloeosporium rot in Kent grown apples is consistent with the apparent absence of tree Neofabraea cankers usually associated with *N. perennans* and *N. malicorticis*. Additionally, a single isolate of *Cryptosporiopsis kienholzii* was recorded in Herefordshire but was absent in the isolates sampled from Kent. The factors which effect the geographical influence on the relative abundance of the *Neofabraea* spp. are, as yet, unknown but may be influenced by climate (wetter and cooler in Herefordshire on average) and/or the agroecosystem (woodland and windbreak species providing an inoculum reservoir).

Studies in other apple growing regions around the world have been carried out and show similar trends. For example in North West USA a similar survey found that *N. alba*, *N. perennans*, and *C. kienholzii* accounted for 6.0, 81.3, and 12.7 % of 150 isolates obtained from apple fruit, respectively, demonstrating that *N. perennans* is the predominant causative agent of Gloeosporium rot overall in this region (Spotts et al., 2009). The study also reported a geographical distribution much like that observed in the UK with *N. alba* being the most common species in Oregon and *N. perennans* was most common in Washington. This exercise is important for monitoring what species are present so that control strategies can be tailored more specifically.

 © 2012 Agriculture and Horticulture Development Board

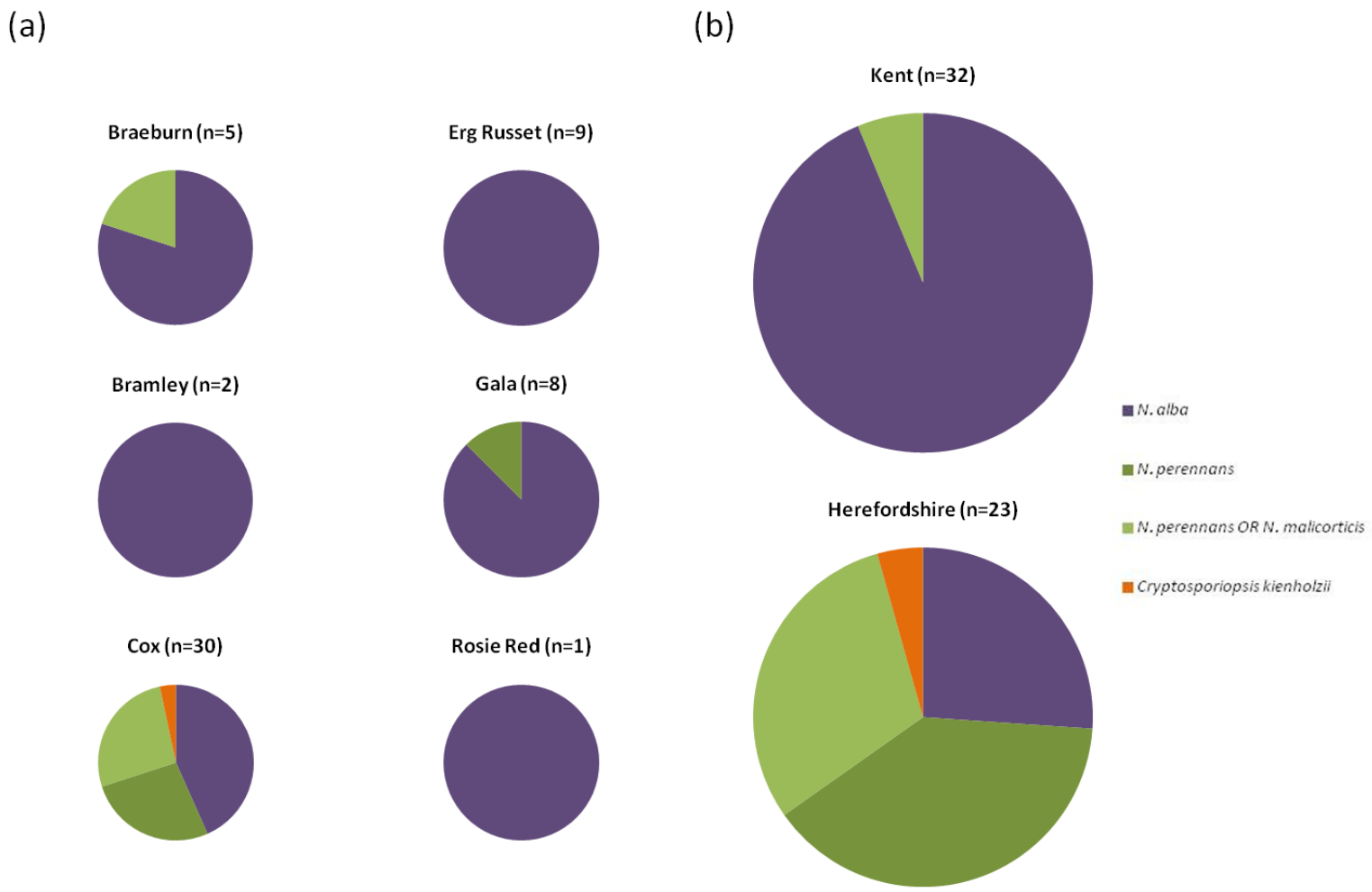

Figure 4. *Neofabraea* species identification of a UK isolate collection as determined by molecular identification and spore morphology by (a) cultivar and (b) region.

 © 2012 Agriculture and Horticulture Development Board

Project 2: Determining the endophytic profile of cultivated strawberry

Introduction

Endophytes are microorganisms (usually fungi or bacteria) which live within the plant without causing apparent disease. Endophytes are receiving increasing attention internationally as they are recognised as agents which can enhance resistance to biotic attack, enhance abiotic stress tolerance and increase growth due to increased solubilisation of minerals and enhanced nitrogen use efficiency. Although the host-endophyte interaction tends to be mutualistic, other 'shades' of endophytism include commensals, temporary residents, latent pathogens or latent saprophytes.

With the recognition of endophytes as important components to the host, much like the microflora of the human gut is important to health, it is important to know what the profile of these organisms are and what influences their survival.

In the last 10 or so years the field of metagenomics, the study of biological content within environmental samples using molecular techniques, has received increasing attention. The recent advances in DNA based molecular techniques have enabled the characterisation of microbial communities within environmental samples, not previously feasible using traditional isolation techniques, which required culturing and morphological/biochemical identification. The application of these techniques to various biological questions has uncovered hidden diversity not visible using traditional techniques, not least in the field of endophytic associations with plants.

Using a metagenomics workflow in development at East Malling Research the endophytic profile of cultivated strawberry will be determined. Knowledge of the endophytic profile of strawberry, and factors which positively or negatively affect it, may have wide implications ranging from pest and disease control, resilience to abiotic stresses and water and fertiliser use efficiency. A potential outcome from this work will be the identification of microorganisms which promote resistance to disease which could be artificially introduced at the pre-planting stage. Latent pathogens (such as *Gnomonia fragariae*) may also be detected in asymptomatic and apparently healthy planting material, enabling a better understanding of the epidemiology of strawberry diseases in commercial crops. Further applications could arise with application of this technique to other horticultural crops such as apple.

 © 2012 Agriculture and Horticulture Development Board

Methods

Validation of metagenomics workflow for Eukaryotic organisms

Validation of the metagenomics workflow for Eukarytotic organisms was necessary to complement the workflow validated previously for prokaryotic organisms, thus enabling the determination of both prokaryotic and eukaryotic organisms within the tissue samples. The consumables required for the validation of the workflow were funded by a Worshipful Company of Fruiterers grant, the personnel time was covered by the fellowship. DNA was extracted from fungi (and oomycetes) commonly occurring on or in association with horticultural fruit crops, quantified and mixed at the proportions described in Table 5. This mix constitutes the synthetic *in-vitro* sample used to optimise and validate the metagenomics workflow.

Table 5. Species profile of the synthetic *in vitro* sample.

Species	Concentration (ng/ul)
Alternaria alternata	20
Venturia inaequalis	20
Podosphaera aphanis	20
Neofabraea alba	20
Botrytis cinerea	20
Sclerotinia sclerotiorum	20
Metarhizium anisopliae	20
Verticillium dahliae	20
Fusarium oxysporum	20
Nectria ditissima	20
Colletotrichum acutatum	20
Rhizophagus clarus	20
Phytophthora cactorum	5
Phytophthora fragariae	5
Phytophthora idaei	5
Phytophthora syringae	5

 © 2012 Agriculture and Horticulture Development Board

Sample preparation

Four different primer pairs (Table 6) were tested to select the best combination for future metagenomic applications. Each primer was modified at the 5' end with adaptors (forward adaptor: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG and reverse adaptor: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA) required for subsequent protocol steps. The synthetic *in vitro* sample was amplified by four different primer pairs using a standard PCR protocol. The PCR was carried out in triplicate and pooled to reduce potential bias in amplification. PCR products were visualised by gel electrophoresis.

Table 6. Primer pairs used to prepare four independent amplicon libraries.

Forward primer name	Forward primer sequence	Reverse primer name	Reverse primer sequence	Expected product size (bp)
>1391f	GTACACACCGCCCGTC	>EukBr	TGATCCTTCTGCAGGTTCACCTAC	~500
>AM_F	GGAAAGATGAAAAGAACTTTGAAAAGAG	>AM_R	TGGTCCGTGTTTCAAGACG	400-600
>ITSI_F	CTTGGTCATTTAGAGGAAGTAA	>ek28_R	ATATGCTTAAGTTCAGCGGG	400-600
>ITSI_F	CTTGGTCATTTAGAGGAAGTAA	>ITS4	TCCTCCGCTTATTGATATGC	400-600

Following a PCR clean-up of the amplicon PCR libraries using Agencourt AMPure XP beads (Beckman Coulter, USA), as per manufacturer's protocol, a barcoded template library was generated by PCR. Identifying Illumina nextera indices, unique to each PCR library allows simultaneous sequencing of multiple samples, i.e. multiplexing (this DNA index is commonly referred as a barcode). Following an index PCR clean-up step, using the Agencourt AMPure XP beads, PCR products were qualitatively assessed using a Fragment Analyser (Advanced analytical, Ames, IA, USA) using the high sensitivity NGS fragment analysis kit (Advanced analytical, Ames, IA, USA). PCR products were also quantitatively assessed using a Qubit 2.0 Fluorometer (Life Technologies, USA). DNA from different samples was then pooled. The unique DNA barcode indices allowed sequences from all samples to be de-multiplexed in subsequent processing. Samples were pooled in such a way to ensure each sample was equimolar (same concentration). The final concentration of the pooled library was 4 nM. The amplicon library was denatured using 1 mM NaOH and diluted to 30 pM. The diluted and denatured amplicon library was then combined with a denatured PhiX library at an equimolar concentration at a rate of 20% to increase heterogeneity of the sample. These samples were then run on an Illumina MiSeq with 300 bp paired end sequencing (V3 chemistry). The protocol followed above was based on the workflow described by Illumina for 16s metagenomic library preparation.

(http://supportres.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).

Data analysis

Sequence data were automatically de-multiplexed by the Illumina MiSeq and then further processed by the QIIME analysis pipeline (Caporaso *et al*., 2010):

(1) Primers removed from sequences;

(2) Quality filtered to remove low quality reads (sequences);

(3) Identify an OTU (operational taxonomic unit, the clustering of sequences by a sequence identity threshold) for each sequence against two international databases: 16S (bacteria) – Silva (Quast *et al.,* 2013), and the UNITE fungal 18S ITS database (Koljalg *et al.* 2005) at 97% similarity;

(4) Store every unique sequence and its frequency for each sample. Data analysis was carried out using MEGAN (the MEtaGenome ANalyser); a tool for studying the taxonomic content of a set of DNA reads.

Optimisation of tissue preparation for the metagenomics workflow

Tissue preparation is an important step to ensure that samples contain true endophytes and are not contaminated with epiphytes living on the surface of the plant tissue. A preliminary experiment was conducted comparing two methods of tissue sterilisation on four tissue types (young leaf, old leaf, petiole and crown (Figure 5)) using strawberry plant material collected from two growing situations (commercial and garden). Reproductive tissues (i.e. flower and fruit) were omitted at this stage due to anticipation of difficulties with DNA extraction.

 © 2012 Agriculture and Horticulture Development Board

Figure 5. Tissues types sampled for method optimisation.

In order to kill epiphytes living on the surface of the sampled tissue two published methods of sterilisation were tested:

(i) Chemical sterilisation (modified from Schulz *et al,* 1993)

Tissue immersed in 100% ethanol for 30 seconds, washed in sterile water, immersed in 33% commercial bleach solution (5% available chlorine) for five minutes, immersed in ethanol for a further 30 seconds and then four separate washings in sterile water. All steps were carried out in 25 ml bijous in a sterile flow hood. Tissue samples were dried on sterilised filter paper disks in a sterile flow hood.

(ii) Physical sterilisation (modified from Lundberg *et al*, 2012)

Tissue sections placed in 50 ml falcons with 25 ml of sterile phosphate buffer (7.18 g of NaH2PO4*2H2O + 22.21 g of Na2HPO4*12H2O + 200 µl Silwet L-77 + 1 L RO water). Tissue was sonicated in a Bandelin Sonorex sonicator amended with ice at low frequency for five minutes (five 30 second bursts followed by five 30 second rests). Tissue samples were dried on sterilised filter paper disks in a sterile flow hood.

For culturing, split Petri dishes were prepared with water agar (WA; 1.2% w/v, technical agar No. 3, amended with 60 mg L^{-1} penicillin G and 80 mg L^{-1} streptomycin sulphate). To collect tissue for DNA extraction and subsequent metagenomics analysis 2 ml eppendorfs containing two ball bearings were prepared.

Three sections of each tissue prepared above were sampled in a sterile flow hood using sterile equipment (scalpel or cork-borer). For leaf material a 6 mm diameter leaf disk was excised, for crown material a 6 mm^2 piece of tissue was excised, for petiole material 6 mm lengths were prepared.

 © 2012 Agriculture and Horticulture Development Board

Excised tissue was cut in half. Half of the tissue was pressed onto one half of the split Petri dish (epiphyte press, Figure 6) the same tissue sample was then placed in the centre of the other half of the plate (tissue sample, Figure 6.). Samples were incubated at 20°C and monitored.

For each sample the other half of the tissue was placed into the prepared eppendorf (material for each tissue class was pooled) and flash frozen in liquid nitrogen. These samples were stored at -80 C°.

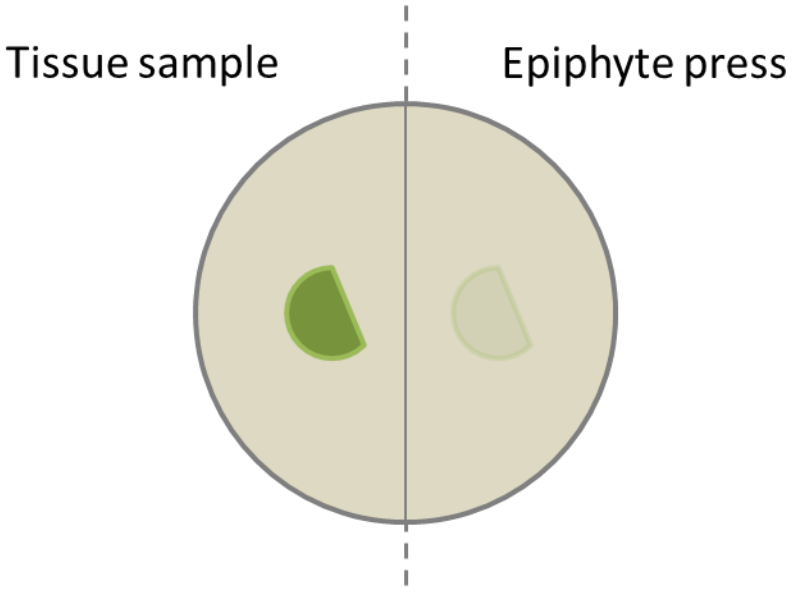

Figure 6. Split Petri dish to determine the effectiveness of the sterilisation techniques.

Results and Discussion

Metagenomic work flow validation

The ITSI-F /ek28_R primer set was chosen for further analysis as it gave better amplification when combined with the barcode attachments used in the Illumina sequencing. PCR amplification using these primers gave a product of ~750 bp, which is consistent with the target region of the rRNA loci including the end of the SSU, ITS1, 5.8S and the start of the LSU region plus the adaptor primers.

Qualitative (presence/absence) taxonomic level analysis of OTU's generated from ITSI-F /ek28_R dataset is presented in Table 7. The data demonstrates the taxonomic resolution achieved using the workflow. Of the 12 fungal species included in the synthetic *in vitro* sample 10 were resolved to the species level, one (*Colletotrichum acutatum*) to the genus

 © 2012 Agriculture and Horticulture Development Board

level and one (*Verticillium dahliae*) to the order level. Of the four species within the *Chromalveolata* (includes the Oomycetes e.g. *Phytohpthora*) none were resolved to any of the taxonomic levels. The fungal species that were not resolved to the species level are present in the database queried. There are many potential sources of bias which may occur throughout the metagenomics workflow which may explain the poor resolution achieved in two of the 12 fungal species in the synthetic *in-vitro* sample. These have been well documented in the literature, for example; differences in cell wall and membrane structures may result in more or less effective DNA extractions from some organisms (Carrigg *et al*, 2007); DNA amplification and sequencing protocols may introduce further bias in the resulting sequences (Temperton *et al*, 2009); post sequencing analysis methods to assign metagenomic reads to taxonomic groupings may also introduce biases (Mavromatis *et al,* 2007). The lack of resolution of the *Chromalveolata* is simply a result of the lack of *Chromalveolata* sequences represented on the UNITE fungal 18S ITS database queried. This has since been addressed as part of another project at EMR by customising the UNITE fungal ITS database to include ITS sequences for Oomycetes.

Table 7. Qualitative analysis of the OTU's generated from the synthetic *in-vitro* sample using the ITSI-F /ek28_R primer set.

Species composition of synthetic in vitro sample	Represented to Class level?	Represented to Order level?	Represented to Family level?	Represented to Genus level?	Represented to Species level?
Alternaria alternata	Y	Y	Y	Y	Y
Venturia inaequalis	Y	Y	Y	Y	Y
Podosphaera aphanis	Y	Y	Y	Y	Y
Neofabraea alba	Y	Y	Y	Y	Y
Botrytis cinerea	Y	Y	Y	Y	Y
Sclerotiniasclerotiorum	Y	Y	Y	Y	Y
Metarhizium Sp	Y	Y	Y	Y	Y
Verticillium dahliae	Y	Y	N	N	N
Fusarium oxysporum	Y	Y	Y	Y	Y
Nectria ditissima	Y	Y	Y	Y	Y
Colletotrichum acutatum	Y	Y	Y	Y	N
Rhizophagus clarus	Y	Y	Y	Y	Y
Phytohpthora cactorum	N	N	N	N	N
Phytohpthora fragarea	N	N	N	N	N
Phytohpthora idaei	N	N	N	N	N
Phytohpthora syringae	N	N	N	N	N

Of the 1,117 OTU's retrieved, following sequencing and analysis pipelines, 41% were assigned to taxonomic groupings (at the order level) which were not present in the synthetic *in vitro* sample. Of those, 95% of the OTU's were assigned to *Boreoplaca ultrafrigida* (Fungi>Ascomycota>Lecanoromycetes>Umbilicariales>Ophioparmaceae>Boreoplaca> ultrafrigida). *Boreoplaca ultrafrigida* is a lichen species described from an extremely

 © 2012 Agriculture and Horticulture Development Board

continental area in Siberia, as such it is highly unlikely that DNA contamination from this species was the cause for this erroneous result. One of the limitations of molecular identification is that the process is heuristic, relying on sequence similarity to sequenced organisms deposited on the databases to determine identity. As such some ambiguous identities are inevitable but often have an explanation. In this case the ITS sequence of *Boreoplaca ultrafrigida* is highly similar to a uncultured ascomycete identified in a Finnish study to "Molecularly profile fungal communities in settled dust of moisture damaged buildings before and after renovations" the sequences of which have been submitted to NCBI (an international database for genomics data). This suggests that contaminants from the air may have been introduced to the sample prior to or during DNA extraction. In future experiments sample contamination can be limited by improving procedures (use of filter tips during DNA extraction protocols and sample preparation in flow hoods).

Quantitative analysis of metagenomic taxonomy data can provide indications of the relative abundance of species within the sampled community. In this experiment all species were represented in the synthetic *in vitro* sample in equal proportions (apart from *Phytophthora* species, which as a whole genus represented the same as the fungal species, see Table 5). The profile determined following the sequencing and analysis pipelines does not reflect the proportions of synthetic *in vitro* sample (i.e. equal representation, Figure 7). The same data is presented graphically in Figure 8. The inconsistencies between expected and actual profiles means conclusions drawn from quantitative data coming from metagenomic data sets should be used with caution. The inconsistencies may result from error introduced during PCR amplification. In future experiments error introduced during amplicon library preparation will be reduced by running more independent reactions for each sample with fewer amplification cycles.

 © 2012 Agriculture and Horticulture Development Board

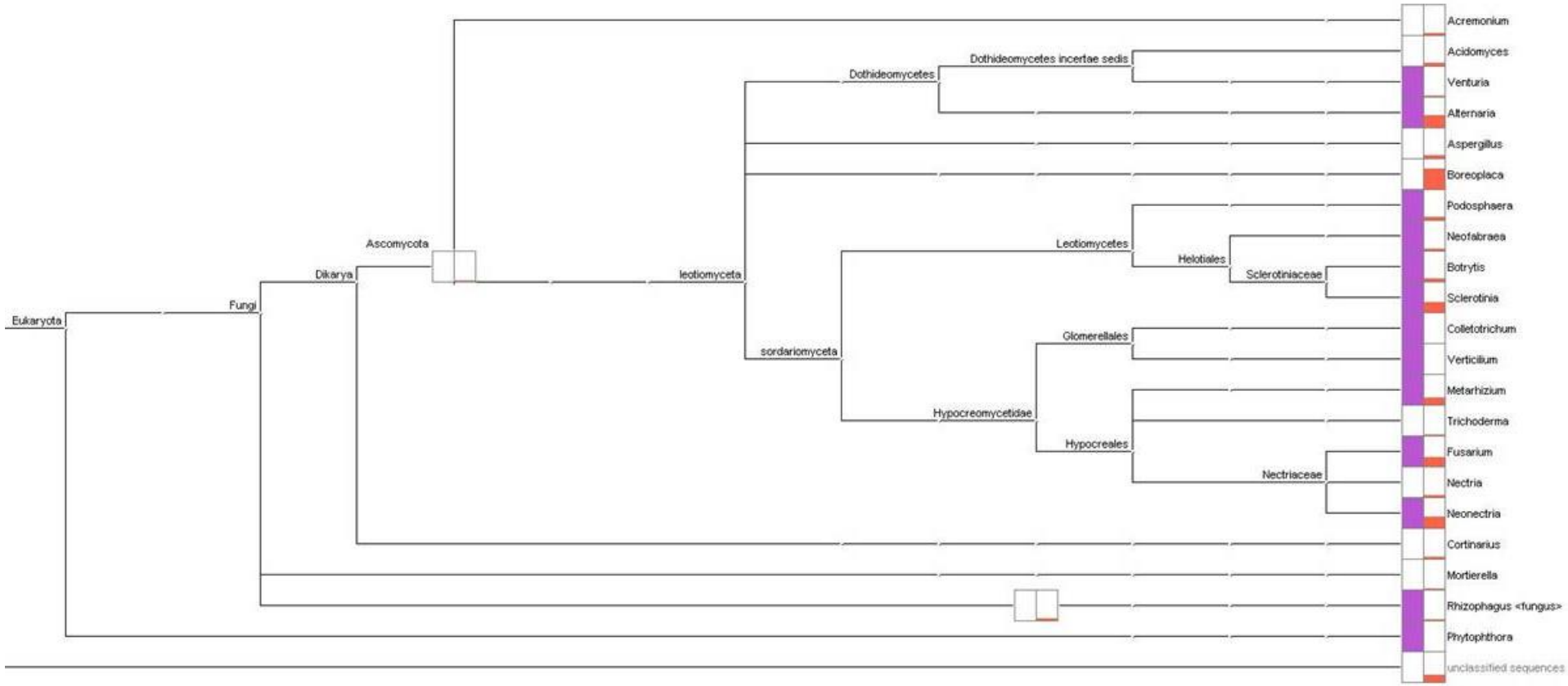

Figure 7. The taxonomic identities at the genus level represented on a cladogram. The coloured bars represent the proportions added to the synthetic *in vitro* sample (purple) and the proportions determined following the sequencing and analysis pipelines (orange).

 © 2012 Agriculture and Horticulture Development Board

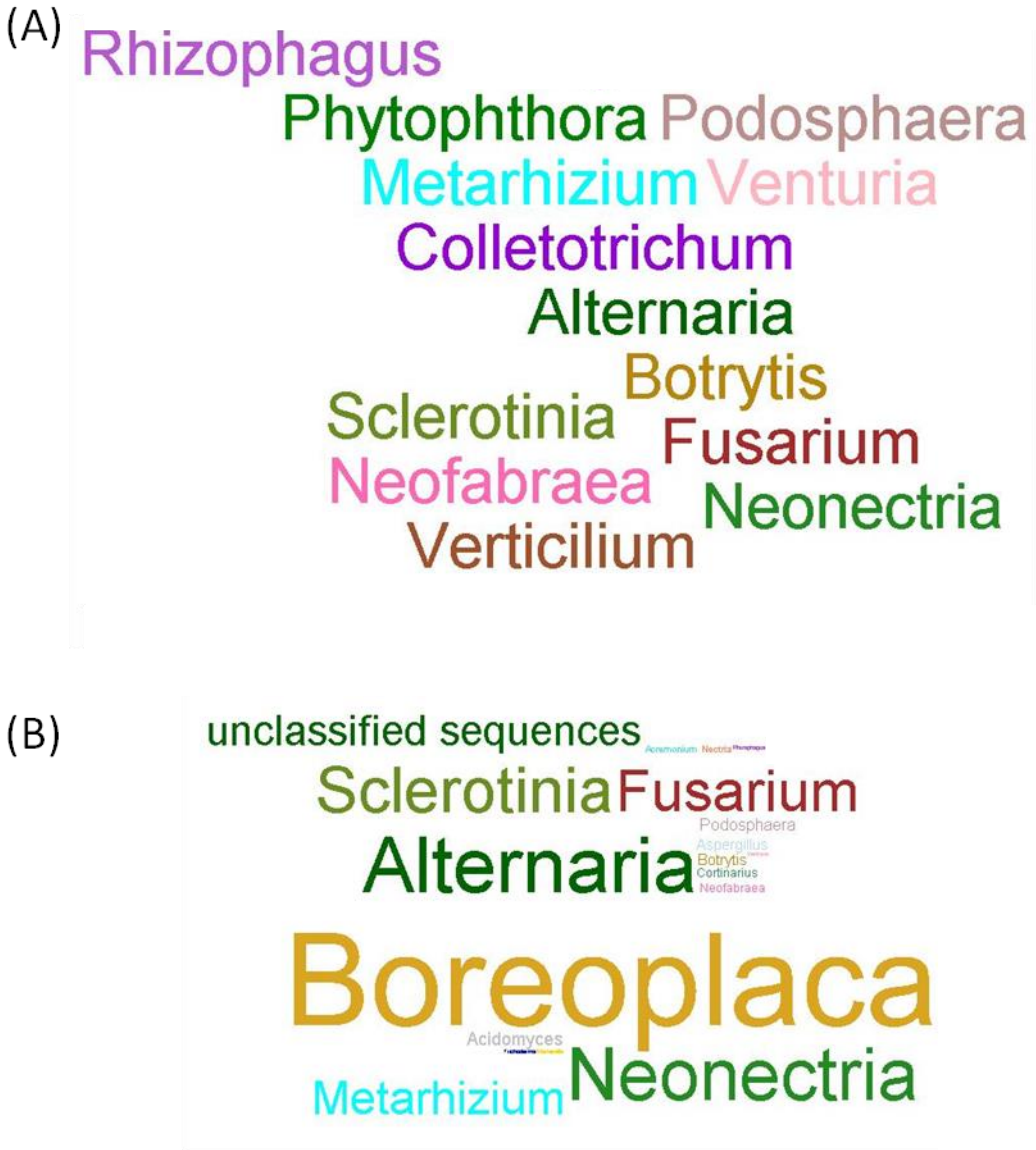

Figure 8. A graphical representation of the taxanomic identities at the genus level of the synthetic *in vitro* sample expected (a) and actual (b). The relative size of the text indicates the relative proportion of the species represented within the sample.

 © 2012 Agriculture and Horticulture Development Board

Optimisation of tissue preparation

Following chemical sterilisation nothing grew on either the epiphyte press or from the tissue sample on any of the tissue samples tested. This suggests that the chemical sterilisation protocol used in this experiment was too severe. The physical sterilisation protocol was not effective. Fungal growth was present on the epiphyte press in addition to the tissue samples (Figure 9) for three of the four tissue classes (no fungal growth was observed on the plates with petiole material).

Physical sterilisation was tested because the physical disruption (by vibration) of the surface dwelling communities is thought to (1) kill and (2) destroy DNA of any of the epiphytes present (Lundberg *et al.* 2012). This is important if sensitive molecular approaches such as the metagenomics workflow are used in downstream applications. Chemical sterilisation was tested because this technique has been used extensively for the characterisation of endophytic communities using traditional culturing methods, where residual DNA contamination from epiphytes is not an issue. Other methods tested subsequently included taking epidermal peels of leaves to remove the epiphytic fraction physically but this method, which is only suitable for leaf material, was not considered practical.

The material collected as part of this experiment is stored at -80°C awaiting sample processing. However further optimisation of tissue preparation is required to ensure that the endophytic fraction is being analysed in isolation. In consultation with prominent researchers in this field refinements of the methods above shall be applied over the winter months, e.g. optimising chemical sterilisation by the serial dipping in different solutions (pers. comm. Alan Gange, Royal Holloway University of London).

 © 2012 Agriculture and Horticulture Development Board

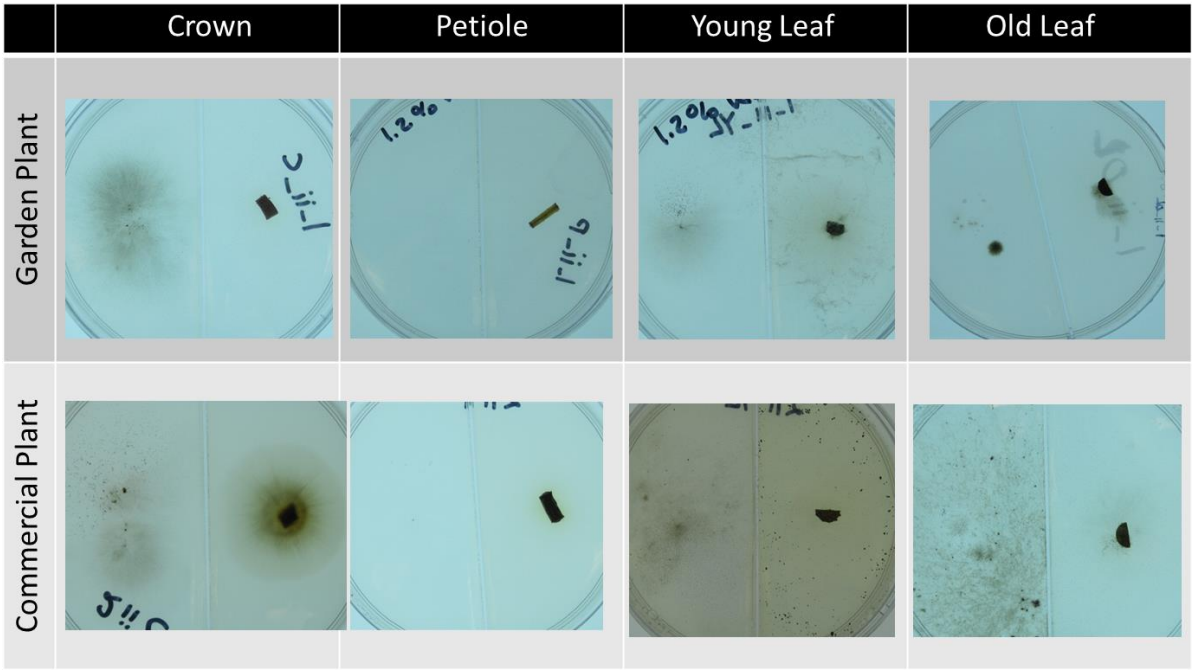

Figure 9. Representative pictures of the fungi cultured from the epiphyte press (left) and tissue sample (right) of four tissue types (crown, petiole, young leaf and old leaf) from plants collected from two growing situations following physical sterilisation. Chemical sterilisation not shown as there was no growth.

 © 2012 Agriculture and Horticulture Development Board

Knowledge and Technology Transfer

Presentation of fellowship project results have been made at various forums:

HDC Agronomists' Day (25/02/14);

HDC Tree Fruit Day (24/04/14), HDC Studentship Conference, York (16-17/09/14);

European Canker Workshop, Sweden (27-29/10/14) and HDC/EMT/HTA funding has been acknowledged accordingly.

A description of fellowship project work will also be featured in the HDC News.

Acknowledgements

The fellowship funds a third of my time enabling me to spend invaluable time shadowing Angela Berrie and developing research areas of my own. Funding was also received from the Worshipful Company of Fruiterers for the consumables required for the metagenomic work flow validation reported in this interim report. I thank the trainers and participants of the TGAC Metagenomics Workshop, Norwich, attendance of which provided much useful information and contacts required for the metagenomics component of the Fellowship Conference. Finally, I acknowledge Justine Perrin, who worked with me on the *Neofabraea* species identification work and Jenifer Kingsnorth, who assisted with the Endophyte work.

References

Berrie, A. M. 1989. Storage rots of apple and pear in South-East England 1980-88: incidence and fungicide resistance. Integrated control of pome fruit diseases, Vol. ll. IOBC Bulletin Xll/6.

Caporaso, J. Gregory et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nature Methods 5: 335- 336

Carrigg C, Rice O, Kavanagh S, Collins G, O'Flaherty V (2007) DNA extraction method affects microbial community profiles from soils and sediment. Appl Microbiol Biotechnol 77: 955–964.

De Jong, S., Lévesque. A., Verkley G. J. M., Abeln E. C. A., Rahe J .E., and Braun G. 2001. Phylogenetic relationships among Neofabraea species causing tree cankers and bull's-eye

 © 2012 Agriculture and Horticulture Development Board

rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. Mycological Research. 105 (6): 658-669.

Koljalg, U et al. (2005) UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist. 166 (3): 1063- 1068.

Lundberg, D.S. et al. 2012. Defining the core Arabidopsis thaliana root microbiome Nature. 488; 86–90.

Mavromatis K, Ivanova N, Barry K, Shapiro H, Goltsman E, et al. (2007) Use of simulated data sets to evaluate the fidelity of metagenomic processing methods. Nat Methods 4: 495–500.

Preece, T. F. 1967. Losses of Cox's Orange Pippin apples during refrigerated storage in England. 1961-65. Plant Pathology, 16: 176-180.

Quast C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Yarza P., Peplies J., Glöckner F.O. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nuclear Acids Research 41: D590-D596.

Saville, R. J. 2013. CP90; Succession planning to sustain the UKs expertise in field and laboratory plant pathology research and development, HDC, Interim report, year 2.

Schulz, B., Wanke, U., Draeger, S. & Aust, H.-J. (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycological Research, 97, 1447–1450.

Spotts R. A., Seifert, K. A., Wallis, K. M., Sugar, D., Xiao, C. L., Serdani, M. and Henriquez, J. L. 2009. Description of Cryptosporiopsis kienholzii and species profiles of Neofabraea in major pome fruit growing districts in the Pacific Northwest USA. Mycological Research 113 (11): 1301-1311.

Sutton, T. B, Aldwinckle, H. S, Agnello, A. M, and Walgenbach, J. F. 2014. Compendium of apple and pear diseases and pests. Second edition. Minnesota, APS Press. pp. 51-53 and 78-79.

 © 2012 Agriculture and Horticulture Development Board

Temperton B, Field D, Oliver A, Tiwari B, Muhling M, et al. (2009) Bias in assessments of marine microbial biodiversity in fosmid libraries as evaluated by pyrosequencing. ISME J 3: 792–796.
Webster, A., Cross, J., Berrie, A. et al. 2001. The Best Practice Guide for UK Apple Production. London, UK: Department for Environment, Food & Rural Affairs.

Wilkinson, E. H. 1954. Fungal rots of apples with special reference to Gloeosporium spp. Annals of Applied Biology, 41: 354–358.

 © 2012 Agriculture and Horticulture Development Board

Appendix - progress against objectives

Objectives

Objective	Original Completion Date	Actual Completion Date	Revised Completion Date
1. Identify and recruit a successor with the most appropriate background to act as understudy to Dr Berrie.	07/11/11	07/11/11	
2. Develop and deliver a training programme to provide the post-holder with skills and experience in the identification of field and laboratory pathology and an ability to conduct and advise on commercial disease management strategies.	06/11/16	ongoing	
3. Facilitate the development of a successor to Dr Berrie through a programme of collaboration (with other technical experts outside EMR), education, demonstration and shadowing, and industry communication to provide the successor with the skills to deliver practical disease management R&D in fruit and other perennial crops.	06/11/16	ongoing	
4. Enable the post-holder to instigate their own sources of income and the delivery of strategic and applied R&D to act as the means to sustain future innovation within commercial horticulture.	06/11/16	ongoing	

 © 2012 Agriculture and Horticulture Development Board

Summary of progress

1. Identify and recruit a successor with the most appropriate background to act as understudy to Dr Berrie. **Completed**

Robert Saville commenced employment at EMR in November 2011. Robert Saville joined EMR having attained his PhD at the John Innes Centre, Norwich working on the dwarfing genes of cereals, their role in cell development and their pleiotropic effects on disease. The combination of experience working with different pathosystems and molecular techniques provide a good foundation to fulfil the subsequent objectives.

2. Develop and deliver a training programme to provide the post-holder with skills and experience in the identification of field and laboratory pathology and an ability to conduct and advise on commercial disease management strategies. **Ongoing**

The training programme during the reporting period has consisted of specific fellowship projects and increasing involvement and management of current and new research projects undertaken in the pathology group. Upon consultation with the industry representative of CP90 (Andrew Tinsley) and the chair of the soft fruit panel (Marion Regan) the fellowship projects were reviewed at the beginning of 2014. The specific fellowship projects are (1) sustainable control of storage rots of apple (2) Determining the endophytic profile of cultivated strawberry. The results of these projects will be reported in the science section below.

In addition to fellowship projects the trainee has had increasing involvement in current and new pathology related projects since Angela Berrie became part-time at the beginning of 2014. Research projects in which the trainee has had involvement during the reporting period include;

HDC tree fruit, European Canker control (New for 2014)

HDC soft fruit, Blueberry dieback

HDC field vegetables, Pumpkin post-harvest losses (New for 2014)

HortLink, SCEPTRE Trials (Apple mildew, Strawberry crown rot and mildew, Raspberry cane diseases)

HortLink, Blackcurrant IPDM

TSB, Biofumigation

TSB, Post-harvest management of plums and cherry

BBSRC, Ash dieback

Commercial trials and consultancy including chemical trials, crop walking and plant clinic.

 © 2012 Agriculture and Horticulture Development Board

3. Facilitate the development of a successor to Dr Berrie through a programme of collaboration (with other technical experts outside EMR), education, demonstration and shadowing, and industry communication to provide the successor with the skills to deliver practical disease management R&D in fruit and other perennial crops. **Ongoing.**

During the reporting period interactions with industry and scientific experts have continued, providing valuable knowledge transfer and collaborative opportunities for the future.

Presentations at the HDC agronomist day (25/02/14) and the HDC tree fruit day (24/04/14) have enabled the communication of research outcomes to the industry. EMR host many tours for various groups providing further opportunities for the trainee to demonstrate the research undertaken in the pathology group at EMR. Tours include; An Austrian grower group (28/07/14), West Sussex Fruit Group (29/07/14), South Tyrolean Nurseryman Association (07/10/14) and the East Kent Fruit Society (11/11/14).

Attendance and presentation at the HDC studentship conference, York (16-17/09/14) developed connections with the next generation of research and development providers serving the horticultural industry.

Interactions with the research community has continued i.e. attendance at the UKPD (UK plant diagnostics group), Alice Holt (19/03/14) and European Canker Workshop, Sweden (27-29/10/14) both of which have led to future collaborative projects.

In the process of developing the proposal for the HDC tree fruit pest and disease IPM tender key industry representatives were consulted providing the opportunity to discuss the key pest and disease issues in the eyes of growers and agronomists based around the UK.

Tom Rouse, a trainee agronomist at Hutchinson's, was hosted at EMR by the trainee for a day to foster links between the industry and the science that serves it.

The trainee has visited other institutions internationally (e.g. Applied Plant Research, Wageningen UR, Netherlands and SILEBAN, Cherborg, France) and hosted international reearchers (Monika Walter and Reiny Scheper, Plant and Food, New Zealand) and industry representatives (Tim Herman, Pipfruit NZ, New Zealand) extending the network of international collaboration opportunities.

4. Enable the post-holder to instigate their own sources of income and the delivery of strategic and applied R&D to act as the means to sustain future innovation within commercial horticulture. **Ongoing**

Lead for a multi-partner consortium proposal to the HDC Tree Fruit Panel for the IPM of Tree Fruit Pests and Diseases tender.

 © 2012 Agriculture and Horticulture Development Board

Contributed to a proposal to the HDC Soft Fruit Panel for the Integrated Pest Management (IPM) of Strawberry Diseases tender.

Assisted in the submission of two Innovate UK Agritech proposals for a systems approach to control European apple canker (unsuccessful at full proposal stage). Involved in continued efforts to secure funding by other means using the partners and ideas from the original proposal.

Appointed and supervise a full time research assistant

Supervised two international students, one worked on the molecular characterisation of *Neofabraea* species present in the UK, the other on screening a *Fraxinus excelsior* (European ash) diversity set for resistance to ash dieback pathogen, *Hymenoscyphus pseudoalbidus*.

Milestones not being reached

All milestones are being reached.

Do remaining milestones look realistic?

All milestones have a realistic completion date.

Training undertaken

In addition to the on-the-job training, detailed above, formal training within the reporting period is as follows;

Bioinformatics Workshop, East Malling (10–12/03/14)

TGAC Metagenomics Workshop, Norwich (8-12/09/14)

Expertise gained by trainees

In addition to the expertise gained from the activities described above the trainee has added to his publication record through major contributions to the following publications;

HDC Pear Crop Walkers Guide

A review of our current knowledge of *Neonectria ditissima* and identification of future areas of research, HDC website

 © 2012 Agriculture and Horticulture Development Board

A review of the literature of the *Neofabraea* species complex, causative agents of Gloeosporium rot in stored apple, HDC website

Other achievements in the last year not originally in the objectives

Appointed to the committee of the East Kent Fruit Society

Visiting Research Fellow at the University of Reading

Organiser of the EMR seminar series

Institute pesticide officer

Changes to project

Are the current objectives still appropriate for the Fellowship?

Fellowship objectives remain unchanged

 © 2012 Agriculture and Horticulture Development Board