Project title:	Progressive die-back symptoms in blueberry: Identification and control.
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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.

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GROWER SUMMARY

Headline

- Three species of Phomopsis and two from the Botryosphaeriaceae family were most associated with severe blueberry die-back.

Background and expected deliverables

The UK blueberry growing industry has expanded rapidly over recent years. Home grown production increased by approximately 50% between 2011 and 2012 as young plantations approached maturity. Sales of fresh blueberries now rival that of raspberries but UK production still accounts for only around 20% of total Summer/Autumn sales and almost none during other periods. Blueberries represent a clear opportunity for increased substitution of UK produce for imports.

When separately recorded, yields per bush are known to reach 6 – 10 kg which would amount to 18 – 30 t/ha if multiplied by standard numbers of plants per hectare. Actual yields per hectare being picked are lower. National output was less than 7 t/ha in 2012/13. Many plantations have yet to reach maturity but pest and disease problems have also been an important cause of lower yields.

There have been an increasing number of reports of growth decline in bushes due to die-back and crown rot type symptoms leading to further investigation by diagnostic laboratories. From 2009-2011, such decline led to severe losses in the west of England where the symptoms led to the grubbing of a young plantation at a farm in Herefordshire.

Affected bushes typically display severe nutrient deficiency symptoms in leaves and premature leaf drop, accompanied or followed by browning or blackening of shoots or whole branches. Symptoms are often limited to one or more branches while other parts of the bush continue to grow almost normally for a while. Affected branches may show signs of limited recovery, with new shoots breaking from previously dormant buds as a growing season progresses, only to fail completely the following spring. In 2010, intensive investigations of the problem on two sites were conducted by FAST LLP using the diagnostic services of Fera. A species of *Phomopsis* was identified in a majority of samples.

The type and progression of symptoms bore much in common with those seen in North American plantations known to be infected with *Phomopsis vaccinii*, a pathogen that is listed as an EU quarantine organism. Using DNA analysis, Fera were able to show that the pathogen was not the same as *Phomopsis vaccinii* but the precise identity of the species was not confirmed.

There were similarities between symptoms reported by blackcurrant growers and studied as part of GSK Project no. 223 (SF12) and those observed in blueberries. SF12 found that *Diaporthe strumella* syn. *Phomopsis ribicola* was consistently associated with blackcurrant die-back.

Die-back problems in gooseberries, redcurrants and grape-vines have also been the focus of recent HDC supported work (SF 131 and two 'expert mini-summit' meetings held at Fera, Sand Hutton). Precise diagnosis has been difficult but *Phomopsis*, *Botrytis, Botryosphaeria*, and *Phytophthora* have been variously implicated.

Diagnosis is made complicated by the knowledge that the presence of a particular fungus on, or within affected shoots, does not mean that it was responsible for causing the disease. Fungi can survive in association with woody plants in a variety of ways, not least as secondary infections of weakened tissue or growing as a saprophyte on already decaying tissue, persisting long after the first cause has perished.

Figure 1. Images of bushes from which *Phomopsis* was isolated (FAST LLP)

Even a cursory inspection of many blueberry plantations is likely to reveal a range of die-back type symptoms. Most common are symptoms associated with blossom, twig and shoot tip infections that progress for a few centimeters only. However it was known that, for example, *P vaccinii* causes such tip and blossom die-back symptoms in addition to the more severe and progressive die-back problems that are the subject of this project. If the same fungi were to be found consistently associated with both aerial symptoms and crown infections responsible for the death of whole branches or plants, this would have important implications for disease management.

The primary purpose of this project was to identify the cause of the aggressive type of dieback and crown rot symptom responsible for rapid decline or death in blueberry bushes. Armed with this information the project might then be able to shed some light on how the problem is spread, within and between sites, and consider methods to manage and control the problem.

Summary of the project and main conclusions

Survey and Sampling

Farms were visited in all the major UK cropping areas including the South East of England, Herefordshire, Devon, Dorset, Northamptonshire, Aberdeenshire and Perthshire. Four different symptom types were observed: tip dieback, tip dieback associated with dead flowers, progressive tip dieback and finally crown death leading to die up.

In the period March – December 2012, 72 plant tissue samples, collected by EMR and FAST, were submitted to Fera for diagnostic work. Sub-samples from some sites were also retained by EMR for separate testing. Samples ranged from whole plants, delivered directly to FERA in York by the researchers, through twigs, roots, fruits and leaves delivered by post or courier. The majority of samples were of plants displaying obvious dieback symptoms. Where fruit, leaves or root samples were submitted this was because symptoms evident on those organs appeared to bear some relation to the die-back symptoms within a plantation. Some additional samples were submitted during 2013.

Field Observations

Following damaging weather conditions during flowering in 2012, a great deal of 'blossom blight' was observed at two Herefordshire sites. *Phomopsis* was clearly identified as being associated with these symptoms, especially on the variety 'Darrow'.

On two sites visited in Scotland, frost damage to flowers was a common problem, with visible *Botrytis* sporing on the dead blossoms and associated shoot dieback. *Phomopsis* was not found in samples from these sites.

Bushes at the Herefordshire sites showed a remarkably high incidence of tip dieback. Most of the sites visited by EMR also showed a high incidence of tip dieback. Whereas this type of symptom is common in blueberry plantations, it is not universal. At various times before and during the period of the project, the authors have separately visited many blueberry plantations, in the UK and overseas, where bushes show few or no obvious tip die-back symptoms, especially of the type shown in Figure 2.

Figure 2. “Antler” symptom

With many fields showing significant shoot die-back but a smaller number suffering from the more serious branch dieback or even bush death, it was important to establish whether there is a link between aerial symptoms and decay symptoms affecting crowns or the base of canes. To that end, samples were distinguished according to whether necrotic symptoms appeared to be the result of basal, tip or side infections. Attempts were made to distinguish ‘die-back’ from ‘die-up’ symptoms by looking for necrotic staining within the otherwise healthy-looking wood below or above the area showing clearly visible external symptoms. Dan Chiuian marked and photographed diseased shoot tips and flowers during the Spring of 2013 and returned on several occasions during the following months to monitor the progress of any die-back symptoms. After a rapid early development, disease development slowed, failing to cause a serious and extensive ‘die-back’ symptom.

Figure 3. Blossom blight, May 2012

Table 1 (below) describes ways by which observed symptoms may develop. We do not know whether basal infections result from the systemic movement of propagules within plant tissues or infection by spores from an external source. Such information is of critical importance for the design of control strategies and the prognosis for plantations already showing significant dead arm like symptoms.

Table 1. Die-Up or dieback?

Tip infection	Lesion	Hyphae grow or other propagule move downwards	**Die-back** of tissue starting at tip and developing downwards
Side infection	Lesion	Girdling of stem by fungal decay or hypersensitive reactions in local tissues	Tissues above the lesion wilt and die due to starvation (**Die-up**)
		Hyphae grow, or other propagule move, downwards	Tissue above the infection point may die if original lesion girdles the stem (**Die-up**)
			Lesions may develop further down the stem or in the crown where opportunities arise (mechanical, chemical, freeze injury?) causing **dead arm** like symptoms
Basal infection	Lesion	Hyphae grow, or other propagule move, upwards, killing tissue	**Dead arm** like symptoms
		Hyphae kill a large enough volume of tissue at the base of shoots or cause a hypersensitive reaction (tyloses etc.)	Tissues above the lesion wilt and die due to starvation (**Die-up**)

Figure 4. Die-back, December 2012

Diagnosis

Ann Barnes and colleagues at Fera followed established protocols for identifying fungi present on the samples by visual diagnosis following dissection and, where appropriate setting up cultures to study them in more detail. The diagnostic focus based on previous experience, was centered on investigating potential fungal problems; previous analysis and initial analysis in the project had not identified any bacterial or viral pathogens.

Not surprisingly a very diverse range of fungi were identified as described in Table 2. *Phomopsis* was the most commonly isolated fungus, present in at least 32% of blueberry samples submitted. The incidence of *Phomopsis* may in fact be greater than this as a further 11% of samples submitted yielded a pathogen that could have been either *Phomopsis* or *Phoma.*

The taxonomy of fungi is complicated and subject to change; in particular both *Phomopsis* and *Botryosphaeria* are under major revision resulting in difficulties in both reliable identification to species and comparison with previously published work. Description of *Phomopsis* species is complicated by the fact that the same species may exist as *Phomopsis* (asexual state) or *Diaporthe* (sexual state). The two forms, although sharing the same DNA, are not morphologically similar. This is further complicated by the fact that the *Phomopsis* state is morphologically very similar to a similar fungus called *Phoma*.

Table 2. Type of fungi isolated from the collected samples (Year 1).

Fungi isolated:	% of all blueberry samples	% of samples when leaf spot, fruit and root samples excluded
Phomopsis/Diaporthe	32	30
Phoma	14	12
Phomopsis/Phoma	11	11
Botrytis	16	14
Fusarium	14	12
Cytospora	9	9
Botryosphaeria	7	7
Phytophthora	9	7
Coniothyrium	5	5
Cylindrocarpon	2	2
Ceratocystis	2	2
Ascochyta	2	2
None	4	2

At least three different species of *Phomopsis* were isolated from symptomatic blueberries based on molecular analysis *(P. viticola, P. eres/conorum and P. theicola).* Not all species satisfied Koch's postulates (a test to confirm pathogenicity) and could not therefore be considered to be primary fungal pathogens. Of the three species, *P.viticola* was the most damaging. Within the genus *Phomopsis* there is a wide range of pathogenicity ranging from aggressive primary pathogens, less aggressive wound pathogens to harmless saprophytes. There are also numerous reports in the literature from other similar woody plants of the ability of *Phomopsis* to live harmlessly and without causing any visual symptoms within woody tissue: A fungus surviving in this way is referred to as an endophyte. External biotic and abiotic factors may act as a trigger for these harmless endophytes to cause disease.

The project also investigated the potential significance of fungi belonging to the family Botryosphaeriaceae as they are potential pathogens of Vaccinium and other similar woody crops. Two species (*Botryosphaeria obtusa* and *Neofusicoccum australe*) were recovered from symptomatic tissue and both isolates satisfied Koch's postulates demonstrating their role as primary pathogens. Again these results are comparable to those found in other countries with evidence of interaction between *Phomopsis* and Botryosphaeriaceae as part of a disease complex.

The project has shown that progressive die-back of blueberries can be caused by more than one species of fungus including those from *Phomopsis/Diaporthe*, *Botryosphaeria/Neofusicoccum and Coniothyrium*. At least some of these species may be able to live within blueberry shoot tissue without causing harm. Even if not already living inside blueberry tissues these species are common inhabitants of plantation habitats, variously growing on such material as old tip and bud infections, decaying prunings and injured flowers. When inoculated directly into wounded tissues the isolates of *Phomopsis* and *Neofusicoccum* collected from sick bushes show only moderate of even weak pathogenicity in laboratory conditions. However, it is also clear from field observations that crown/basal infections may extend sufficiently to cause wilting and starvation of branches. There are an increasing number of studies showing that *Phomopsis/Diaporthe* and *Botryosphaeriaceae* can survive within plants without causing disease but may also cause disease when their host becomes stressed by other factors.

Interestingly, during pathogenicity testing, Fera discovered that symptomless plant material supplied for the harvesting of shoots used for pathogenicity tests was harbouring *Phomopsis,* apparently living as an endophyte within the plant tissues. Shoots wounded during the inoculation process used for pathogenicity testing developed lesions that were found to contain a species of *Phomopsis* that was not the one, for which a pure isolate had been prepared and inoculated into the wound. Some months after the experimental work was completed, three of the 20 stock plants that had been grown in isolation at Fera were found to have died. *Phomopsis* was isolated from one and *Coniothyrium* from another which again illustrates the potential of healthy looking young plants to be carrying potentially lethal pathogens.

Main conclusions

Throughout the UK, blueberry plantations contained plants with die-back symptoms. These ranged from blossom blight and tip dieback to a more serious and frequently lethal crown infection leading to branch death or plant loss.

- No evidence of any bacterial or viral pathogens was detected; no consistent fungus was isolated from the various symptoms, but a diverse range of potential fungal pathogens including *Phomopsis, Botryosphaeria, Botrytis* and *Coniothyrium* were isolated.

- *Phomopsis* was the most commonly isolated fungus from about a third of symptomatic plants; three different species were identified of which *P viticola* was the most damaging. Importantly, no evidence of the EC quarantine listed pathogen *P. vaccinii* was found.

- Two species of Botryosphaeriaceae were isolated and both also proven to be pathogenic.

- The species (*Phomopsis ribicola*) found by HDC Project SF12 to be most associated with severe dieback in blackcurrants, was not identified in blueberry samples for which diagnostic work was taken to the level of species identification.

- In-field symptoms did not provide a reliable indication of the species of fungal pathogen involved.

- UK field experience is that bushes exhibiting 'die up' symptoms due to crown death tend not to recover, becoming progressively weaker over a period of years.

- The speed with which bushes succumb to serious dieback symptoms appears to be controlled by factors other than the simple pathogenicity of the infecting fungi. There may be interaction with other pathogens in a disease complex or an over-riding influence of environmental and cultural factors. Work is needed to investigate the effect of chemicals on disease expression, in particular the plant strengtheners, elicitors and growth promoter products that are reported to increase resistance of plant to diseases.

- *Phomopsis*, perhaps living as an endophyte, was isolated from symptomless young plants used in the pathogenicity tests. Of the 20 symptomless plants provided for the pathogenicity tests, three died within six months from which *Phomopsis* was recovered on one occasion in addition to *Coniothyrium*.

- It is not known whether it is practically possible to eradicate these fungi from propagation sites, mother stock or cropping plantations nor whether eradication would make plants more or less susceptible to later disease infections. Further work on this subject is required.

- Confirmation that *Phomopsis* and species from the *Botryosphaeriaceae* family are important in the development of blueberry dieback symptoms should be used to inform fungicide choice.

Further work

More research is needed to investigate the relationship blueberry plants and *Phomopsis*, *Coniothyrium* and the *Botryosphaeriaceae*. Scientists need to find out how these fungi are able to survive within apparently disease free plant material, what stress factors are responsible for rapid and dangerous expansion of established basal lesions (causing progressive dieback) and by what means they might be eradicated from bushes and/or plantation or propagation environments. The findings of the blackcurrant project SF12 indicated that commonly available systemic fungicides may not be effective in this respect.

It is important to establish whether fresh plants may be planted into growing media from which infected bushes have been removed. If growers were simply able to rogue out plants showing symptoms of progressive dieback at any time during the development of plantations and to replace those plants with fresh material into the same growing media (pots or soil) with a minimum of additional inputs, this disease would immediately be made more manageable during the early years of plantation establishment.

It has become clear from contacts made and recent scientific reports, that the type of disease complex presented by progressive blueberry dieback has much in common with those observed in many other crops and caused by the same groups of fungi. We need to expand our knowledge of relevant work being carried out by pathologists working in other crop groups.

Information on the identity of the species of fungi associated with dieback may be used to inform decision making about fungicide selection.

Financial benefits

The establishment cost for a new blueberry plantation is particularly high. Fields are planted with at least 3,000 plants per hectare and often more when soil-less systems are used. Plants are supplied in pots at a cost of up to £3.00 per plant. Most soil grown plantations require expensive amendment of the soil using sulphur and organic mulches. Pot grown

blueberries incur the cost of pots, compost/growing media and supporting/drainage infrastructure. The final cost of establishment, may be as high as £6.00 per plant so it is of critical importance that they do not fail before achieving a return. The loss of plants to die-back disease can have a substantial impact on profitability. Sick plants demand extra husbandry work, produce small fruit and make no contribution to paying for sprays and other field costs. The situation is made harder still when the cause and source of infections are uncertain. The risk of cross infection from replanted soil, composts and other materials cannot be quantified or properly addressed.

Identification of the species most likely to cause progressive die-back of blueberries has already enabled agronomists and growers to focus on strategies known to reduce disease pressure (hygiene, fungicide selection, spray timing) from those species during fruit production and in propagation.

Action points for growers

Several fungi were found to be associated with blueberry dieback and death. The species most commonly found may be described as 'wound pathogens', tending to take advantage of weakened or damaged tissues including flower parts, frost damaged shoots and shoots damaged by such things as vine weevil larvae and pruning.

Blossom and shoot tip infections caused by *Botrytis cinerea* and several *Phomopsis* species frequently appear but it was found that they tend not to progress into more severe die-back symptoms. These species, together with *Botryosphaeria (Neofusicoccum)* and *Coniothyrium* species identified in some samples are all capable of infecting blueberry shoots. Although it appears that the relationship between these fungi and disease development is a complicated one, the following guidance can be given to growers:

- Prune or snap out twigs showing any kind of dieback symptom during the dormant season as it is possible these are a source of inoculum. Remove the prunings, ideally from the plantation but at least from around the base of bushes. If left on the ground they may still provide inoculum because the fungi are able survive successfully in dead material.

- During highest risk periods (eg. blossom, frost/hail events, freeze damage, planting/transplanting) select fungicides with a sufficiently broad spectrum of activity to

control all of *Botrytis*, *Phomopsis* and the *Botryosphaeriaceae*. It should be noted that, in America, the days between bud break and petal fall is regarded as the highest risk for infection by *Phomopsis* species.

- Avoid basal injuries to young plants during propagation and after planting out. Potential causes of basal injury include vine weevil larvae, pruning, rough handling, fertiliser scorch and freeze injury. High risk situations include permanently moist compost in contact with wounds, humidity (growing and storage) and harsh weather between bud break and flowering. Weak basal shoots that are typical of young plants during propagation and on arrival at farms for planting out are vulnerable to infection, often showing symptoms that are characteristic of the severe die-back symptoms studied for this project. They should be regarded with suspicion. All possible measures should be taken to prevent the infection of basal shoots by dieback fungi, both in nurseries and after planting out.

SCIENCE SECTION

Introduction

Die back of shoots and a crown-rot symptom had been noted in blueberry plantations throughout the UK over several seasons. More recently the problem appeared to increase, particularly in the West of England. At one farm (farm code E7X), approximately 90% of plants showed some symptoms and >3% of plants were completely killed. At a second farm (farm code H2R) more than 5% of plants were lost in 2010 with > 90% of the cultivar 'Duke' showing some symptoms. In the summer of 2011 the problem at farm code H2R increased, with around 30% of a plantation established in spring 2010 showing severe dieback. By July 2011 virtually all bushes were showing some evidence of infection and the whole plantation was subsequently grubbed out. The fungus *Phomopsis* is the only pathogen consistently isolated from affected bushes at farms E7X and H2R. Similar symptoms are apparent on other blueberry farms, although not consistently resulting in such significant mortality.

In the autumn of 2011 severe losses were reported by another major producer in the West of England (farm code H4R). A Fera diagnostic report, dated 27/10/11 and referring to samples from farm H4R, stated that: "We confirmed the fungus that we consistently isolated from the lesions as *Phomopsis",* a result that confirmed an identical in-field visual diagnosis based on external symptoms and a rough dissection of affected bushes by FAST advisors. The most common symptom observed was leaf loss and rind browning at the shoot tips, which may or may not develop into more significant wilting and dieback. Often this dieback was limited to one or more branches while other parts of the bush continued grow to almost normally in the same growing season. Frequently, affected branches showed signs of limited recovery but failed completely 6 -12 months later. Some of the above ground symptoms closely resemble those of *Phytophthora,* which is a known problem in blueberry. However, in most of the cases the roots are often relatively healthy until the affected part of the crown has completely failed. In most instances *Phytophthora* has not been isolated from affected bushes or their roots.

In 2010 Fera used DNA analysis to show that the *Phomopsis* found at farms H2R and E7X was not the EU quarantine listed species, *Phomopsis vaccinii*, but the precise identity of the species was not confirmed. Fera has extensive experience of diagnosing and identifying *Phomopsis* using morphological and molecular techniques, not only on *Vaccinium* but also on other woody species such as vines (Vitis trunk disease and die-back).

There appear to be similarities between symptoms reported by blackcurrant growers and studied as part of Project no. SF 12-223 and those observed in blueberries. SF 12-223 has identified that *Diaporthe strumella* syn. *Phomopsis ribicola* has been consistently associated with the blackcurrant problem referred to above, however there was no current evidence that the causal agent is the same as that responsible for blueberry dieback (personal communication from Charles Lane, Fera, November 2011).

Although *Phomopsis* species were more consistently found in samples from farms H2R, H4R and E7X, similar symptoms were associated with *Botryosphaeraceae* and *Coniothyrium* at other sites. Indeed, these fungi were also found in some samples submitted from farms H2R, H4R and E7X.

In other parts of the world blueberries are affected by a wide range of pathogens, most of which are not thought to present a serious threat to production in the UK because of climatic or cultural reasons. However the sudden and extensive onset of symptoms observed at farms H2R, H4R and E7X, and associated with a species of *Phomopsis,* was of great concern. However, although *Phomopsis* had been consistently isolated from samples taken from these farms, other fungi were also present. It was considered likely that dieback symptoms might be caused by more than one fungus, perhaps interacting with each other and with abiotic stress factors (drought, freezing damage, wounds caused by insects etc.). Communication with teams studying dieback disease of blueberries and other perennial crops (particularly grape vines and currant bushes) in Germany, USA, Holland, Poland, France and Chile has revealed that similar symptoms are common to these crops in many countries. As in the UK, a range of fungi have been associated with the symptoms. Broadly speaking, the range of genera found is similar but the species, especially of *Phomopsis* are more variable.

The purpose of SF 132 was therefore to:

(1) Identify the cause of the aggressive type of dieback and crown rot symptom responsible for rapid decline or death in blueberry bushes;

(2) To provide better information to growers and diagnosticians wishing to causal organisms or factors;

(3) Discover how the problem is spread within and between sites and

(4) To move towards the development of methods for management and control of the problem.

Materials and methods

Site visits

Year 1:

During 2012 visits were made to blueberry sites in England and Scotland, particularly to those where a dieback problem was known to be present. At each visit the crops were generally viewed for symptoms of dieback. The dieback symptom types were recorded and photographed and samples taken. All samples were sent to Fera at Sand Hutton, York for diagnosis. Details of the sites visited are shown in Table 3.

Year 2:

Site visits were scaled back so that resources could be focused on laboratory based work carried out by Fera at Sand Hutton. However EMR and FAST re-visited selected sites to observe symptom progression. Dan Chiuian of FAST marked symptoms at two sites, returning on several occasions to compile a photographic time-series.

Fungal isolation and identification

Seventy-two plant tissue samples had been submitted to Fera for diagnostic work in 2012. Sub-samples from some sites were also retained by EMR for separate testing. Samples ranged from whole plants, delivered directly to Fera, through twigs, roots, fruits and leaves. Where fruit, leaves or root samples were submitted this was because symptoms evident on those organs appeared to bear some relation to the dieback infection or expression process. Three tissue samples from willow shrubs growing close to diseased blueberries in Devon were also tested in response to concern, expressed by a grower, that a disease showing on the willows was related to death of his blueberry plants.

Ann Barnes and colleagues at Fera followed established protocols for identifying fungi present on the samples by visual diagnosis following dissection and, where appropriate setting up cultures to study them in more detail. Charles Lane (Fera) studied the results and assembled a spreadsheet in which he listed the predominant fungi found, by sample.

Samples were allocated a code in the field and at Fera. A very large number of photographs were taken. Fera also used molecular techniques to confirm the identity of fungi where

appropriate. A literature search was also carried out to improve understanding of the incidence and possible causes of dieback diseases in other countries.

Table 3. Blueberry sites visited in 2012

Grower/contact code	Area	Blueberry crop type
B9H	West	Field grown. Mainly open field some under polytunnel
M11E	East	Open field on raised beds
M10E	East	Open field
P8H	North	Mainly field grown. Some open field, mostly under polytunnel
A6B	North	All potted under poly tunnels
P5E	East	All potted. Some open field, some in poly tunnels
C1O	East	Field grown under poly tunnel
G3U	East	All potted. Mostly open field
H4R	West	All potted. Some open field, some in poly tunnels
H2R	West	Potted and soil grown. Mixture of polytunnels and open field.
E7X	West	Soil grown, open field

Laboratory samples

Detailed examinations were made of the branches, crowns and roots of affected plants and any symptoms of dieback, lesions or decay noted. Photographs of samples and symptoms were also taken. The plants were subsequently tested using the following techniques:

Aseptic isolation

Isolations were made onto nutrient agar from areas of decay or dieback affecting the branches, crown or roots. Small pieces of affected woody tissue, ideally taken from the leading edge of the decay (the area where diseased tissue meets adjacent healthy tissue), were surface sterilised for between 2-3 minutes by immersing them in a solution of 10%

bleach. The tissue pieces were then blotted dry on filter paper before being placed in Petri dishes containing the nutrient agar. Where decay affected the internal pith of branches, small pieces of pith were removed and placed onto the agar without surface sterilisation.

The agar used for the initial isolations was usually quarter strength potato dextrose agar with the addition of the antibiotics penicillin and streptomycin (¼ PDA + P&S). Occasionally, full strength potato dextrose agar without antibiotics (PDA) was used. Root and crown tissues were also isolated onto the *Phytophthora*-selective agar PARPH.

Humid incubation

Sections of branches, roots or crowns with decay were placed onto damp paper towel in sealed plastic boxes and incubated at ambient temperature, to encourage growth and spore production by any potential fungal pathogens.

Floating technique

Decayed crown and root tissues were tested for the presence of *Phytophthora* using an incubation technique. The plant tissues were immersed in Petri's solution in Petri dishes. These were incubated for 3-10 days and microscopically checked every few days for *Phytophthora* sporangia.

Lateral flow devices (LFD's)

Decaying root and crown tissues were tested for the presence of *Phytophthora* using lateral flow devices (LFD's) specific for this fungus. The devices have been developed and are supplied by Forsite Diagnostics Ltd, Sand Hutton, York, YO41 1LZ. Plant tissues containing *Phytophthora* react to produce a blue line in the test.

Laboratory Identification of potential pathogens

Potential pathogens were identified using a combination of colony and spore morphology (checked using visual and microscopic examination), and molecular testing (using DNA sequencing).

Molecular Characterisation (Year 1)

The DNA sequencing technique was as follows: pure cultures of the fungus to be sequenced were extracted using the Nucleospin plant II kit. The extracted DNA was amplified using primers specific for the nuclear ribosomal RNA gene (a commonly sequenced gene for fungi), and run on an agarose gel to ensure that only one product had

been amplified. The PCR product was purified for sequencing using the Qiagen PCR purification kit, and sent to MWG Eurofins sequencing service in Germany (Eurofins MWG Operon, Anzinger Str. 7a, 85560 Ebersberg). The sequencing results obtained were analysed in MEGA (an alignment programme) in order to create a consensus sequence, which was then compared with Genbank, a library of known DNA sequences.

Molecular Characterisation (Year 2)

Representative isolates were purified and maintained in the Fera fungal culture collection and sub-cultured onto PDA when required. Standard DNA extraction and purification techniques were used and DNA was amplified using primers specific for the ITS (internal transcribed spacer) and EF (translocation elongation factor) genes. The sequencing results obtained were analysed in MEGA (alignment program) in order to create a consensus sequence, which was then compared with data from Genbank, a library of known DNA sequences. Phylograms for ITS sequence and EF were produced and unknown isolates compared with known isolates with reference to published work concerning characterisation of *Diaporthe* from stem canker of Blueberry in Chile (Elfar, et. al, 2013). A concatenated tree was obtained combining both ITS and EF data.

Colony growth rate

Representative isolates were grown on 4% PDA at room temperature (22°C) with a 12 hours light/12 hours dark regime. A small agar plug was aseptically removed from the margin of the colony and placed on the surface of another PDA plate. Plates were incubated for 7 days and radial growth determined along 2 axes (x and y) with 4 replicates per isolate. The mean of each axis was calculated.

Spore measurements

Representative isolates were sub-cultured onto PDA as described above and incubated at 25°C for a period of up to 6 weeks. Cultures were checked regularly for the presence of fungal fruiting bodies which were removed aseptically to check for the presence of asexual spores (conidia). Mature fruiting bodies were mounted in lactoglycerol and examined microscopically under a compound microscope. The length and breadth of both spore types (alpha and beta if present) of 50 spores was selected at random and measured. The range and mean length and breadth were calculated and results are presented in the Table. Not all isolates produced conidia in sterile culture.

Koch's postulates

Twenty blueberry plants (cv. 'Duke') were obtained from a commercial grower, placed in 10 L pots and maintained with drip feed irrigation in a raised bed at Fera. Plants were grown on for several months with both flowers and fruit removed to encourage vegetative growth. Plants were checked on a regular basis for signs of damage or disease. In August, healthy looking current seasons terminal shoots were removed and cut into 10 cm lengths. These were then surface sterilised by immersing in 70% industrial methylated spirits for 5 minutes and then air dried in a sterile environment. They were then placed in a clean incubation chamber (small clean disposable box containing dry absorbent paper). A small incision was made at the base of each stem to create a flap of tissue into which a small piece of agar, removed aseptically from each test culture was placed. A sterile piece of damp cotton wool was then placed on the inoculation site which in turn was sealed with Parafilm. At least three replicates were prepared for each isolate. Material was incubated at room temperature (22°C) with 12 hours light/12 hours dark and checked on a regular basis for symptoms and saprophytic colonisation.

After 3 weeks incubation, each stem was examined superficially for the presence of lesions and then aseptically dissected longitudinally to check for the presence of any internal necrosis. Isolations onto 1% PDA occurred to monitor for the presence of *Phomopsis*. Isolates were cultured to confirm the presence of *Phomopsis* and representative isolates identified using molecular techniques as appropriate. The number of stems out of the total set up with confirmed *Phomopsis* or *Botryosphaeria*, as appropriate, was calculated and results are presented in the Tables.

Molecular characterization

Representative isolates were purified and maintained in the Fera fungal culture collection and sub-cultured onto PDA when required. Standard DNA extraction and purification techniques were used and DNA was amplified using primers specific for the ITS (internal transcribed spacer) and EF (translocation elongation factor) genes. The sequencing results obtained were analysed in MEGA (alignment program) in order to create a consensus sequence, which was then compared with data from Genbank, a library of known DNA sequences. Phylograms for ITS sequence and EF were produced and unknown isolates compared with known isolates with reference to published work concerning characterisation of *Diaporthe* from stem canker of blueberry in Chile (Elfar, et. al, 2013). A concatenated tree was obtained combining both ITS and EF data.

Results

Field symptoms

Four types of symptoms were observed in the blueberry crops visited:

A) A limited tip dieback (Figure 5),
B) A tip dieback associated with flower infection (Figure 6),
C) A progressive tip dieback (Figure 7)
D) A ‘die-up’ originating as a crown rot (Figure 8).

All sites visited had tip dieback. In some cases the incidence was relatively low and was not causing significant problems. However, in a number of plantations ‘die-up’ type symptoms were present and had resulted in the death of part or whole bushes. Symptoms observed at the various sites and fungi isolated from them are shown in Table 4.

Figure 5. Tip dieback

Figure 6. Tip dieback associated with flower infection

Figure 7. Progressive tip dieback

Figure 8. Die-up

Where possible symptoms presented by samples received by Fera were photographed before sub-samples were removed for diagnostic work with a view to compiling a visual guidebook to assist growers and agronomists. Unfortunately similar field symptoms were associated with several different species and although some general diagnostic guidance for field workers can be derived from our results, specific and reliable visual diagnosis is not possible. See Appendix 1 for examples of photographs and some general comments.

It is possible that distal (tip) infections generate propagules that find their way down to the base of canes and into the crown of bushes. However, our general observation has been that the problem results from the expansion of a lesion (necrotic tissue) within the crown of bushes rather than directly damaging extension of a lesion downward from a distal point. During both the survey and follow up visits the team photographed symptoms presenting in the field. In some instances symptoms were re-visited several times. A pictorial time-series from this work can be found in Appendix 2 and are referred to in the discussion. During the period of the project, symptom development at the frequently visited Farm code H4R showed little or no progression. After the most weakened plants were grubbed and replaced, into fresh growing media, the plantations have grown well. By contrast plantations at Farm code M10E, saw serious decline during the same period.

Table 4. Symptoms present at blueberry sites visited and fungi isolated in 2012

Farm code	Symptom type			
	Tip dieback	**Dieback from flower**	**Progressive dieback**	**Crown rot with die-up**
B9H[1]	Yes ?	Yes ?	No	No
M11E[1]	Yes *Phoma/Phomopsis, Fusarium, Cytospora, Pestalotia*	No	Yes ?	Yes *Ceratocystis, Phoma, Phytophthora, Cylindrocarpon, Phoma*-like
M10E[1]	Yes ?	No	Yes ?	Yes ?
B9H[1]	Yes *Botrytis, Phomopsis, Botryosphaeria*	Yes *Phomopsis*-like	Yes *Cytospora, Phomopsis*-like	No
A6B[1]	Yes *Botrytis*	Yes ?	Yes *Cytospora, Botrytis*	Yes *Botrytis*
P5E[1]	Yes *Phomopsis/Phoma,Phomopsis-like, Cytospora-like*	Yes ?	No	No
C1O[1]	Yes *Botryosphaeria*	Yes *Phomopsis, Fusarium*	Yes *Phomopsis, Botryosphaeria*-like	No

Farm code	Symptom type			
	Tip dieback	**Dieback from flower**	**Progressive dieback**	**Crown rot with die-up**
G3U[1]	Yes ?	No	No	No
H4R[1 & 2]	Yes *Phomopsis/Phoma*	Yes ?	Yes ?	Yes *Phomopsis, Coniothyrium, Phytophthora*
H2R[1 & 2]	Yes *Phomopsis*	Yes *Phomopsis*	Yes *Phomopsis, Phomopsis/Phoma, Ascochyta, Diaporthe-*like*, Cytospora Phoma, Fusarium,*	Yes *Phomopsis/Phoma, Botryosphaeri, Coniothyrium, Fusarium, Phomopsis*-like*, Phoma*
E7X[2]	Yes	Yes *Phomopsis*	Yes	Yes *Phomopsis, Fusarium*

[1]Samples taken and descriptions provided by Angela Berrie/Robert Saville, EMR

[2]Samples taken and descriptions provided by Graham Moore/Dan Chiuian, FAST

When isolated from V*accinium* subjects, *Phoma* and *Phomopsis* are very difficult to distinguish using standard visual techniques because the conidia produced share the same appearance. Where the identification is given as "*Phomopsis/Phoma"* (Tables 3 and 5) diagnostic work did not progress to a point at which the two could be properly distinguished.

Samples submitted prior to the start of the project, from three farms severely affected by progressive dieback symptoms had consistently shown the presence of *Phomopsis* at the base of diseased canes or in affected crowns. 32% of blueberry samples submitted for the project yielded a clearly identified *Phomopsis* or *Diaporthe*. A further 11% yielded a pathogen that could have been either *Phomopsis* or *Phoma.*

Following damaging weather conditions during flowering in 2012 a significant incidence of 'blossom blight' was observed at the two Herefordshire sites. *Phomopsis* was clearly identified as associated with these symptoms. The symptom that was most obvious on the cultivar 'Darrow'. These symptoms did not progress into the kind of more serious or fatal condition which was the primary subject for investigation.

On two sites visited in Scotland frost damage to flowers was a common problem with visible *Botrytis* sporulating on the dead blossoms and associated shoot dieback. *Botrytis* was also consistently isolated from samples of dieback from these sites (Table 5)

Fungal isolations

Isolations from the various samples submitted to Fera resulted in a range of fungi including *Phomopsis, Phoma, Fusarium*, *Cytospora, Coniothyrium, Botrytis* and *Botryosphaeria* (Table 4). No consistent fungus was isolated from the various symptoms, although *Phomopsis* was the most commonly isolated genus, perhaps especially from flower related dieback.

A brief synopsis of the predominant fungi found on the samples is provided in Table 4 and Table 6 provides an impression of the frequency by genus.

Table 5. Brief synopsis of predominant fungi found on *Vaccinium* dieback samples

Phomopsis (*Diaporthe)*	Recognised pathogen causing cankers, twig blight, fruit rot etc. numerous species recorded on this post with variable pathogenicity.
Phoma	Common opportunistic pathogen on a wide range of woody hosts, limited evidence of this being a pathogen on *Vaccinium* but quite a complex group of fungi of varying significance on other hosts.
Cytospora	A large genus with numerous species, commonly found on woody material but no specific record on *Vaccinium*
Ceratoscystis	A large genus with numerous species, commonly found on woody material but no specific record on *Vaccinium*
Botrytis	A common and recognised problem on *Vaccinium*
Coniothyrium	A common opportunistic pathogen on a range of woody plants.
Botryosphaeria	A recognised problem on *Vaccinium*
Fusarium	Most probably an opportunistic pathogen. Has been associated with dieback before but properly as part of a disease complex

Table 6. Pathogen incidence as a % of all isolations (many samples harbored more than one pathogen)

%	All blueberry samples	Leaf spot, fruit and root samples excluded
*Phomopsis/Diaporthe**	32	30
Phoma	14	12
Phomopsis/Phoma	11	11
Botrytis	16	14
Fusarium	14	12
Cytospora	9	9
Botryosphaeria	7	7
Phytopthora	9	7
Coniothyrium	5	5
Cylindrocarpon	2	2
Ceratocystis	2	2
Ascochyta	2	2
None	4	2

**Diaporthe* is the sexual stage of *Phomopsis*.

Characterisation and pathogenicity of *Phomopsis* and *Botryosphaeria* Isolates (Yr 2)

Phomopsis

Colony growth rate

Growth rate was measured for isolates with considerable range in the mean radial growth measurements from 1.5 to 6.5 mm over a seven-day period. Although based on a limited number of isolates there was a similarity (e.g. isolates 8 and 9) as well as considerable variation in growth rate within species (e.g. isolates 2295 and 2360) as well as between species (e.g. isolates 8 and 21385).

Spore measurements

Not all isolates (e.g. isolates 9, 2773 and 21389) produced conidia. In addition isolate 2295 only produced alpha and not beta conidia. The mean length of alpha conidia ranged from 5.25 to 6.95µm and with from 1-2.5 µm; beta conidia ranged from17.5 to 35 µm long and 1-1.1 µm long. Measurement compared favourably to published descriptions, although spore diameter ranges are quite broad.

Koch's postulates

The results were highly variable with little consistency but illustrated a very interesting element to the project. The initial round of Koch's postulates superficially demonstrated at times considerable damage to the stem portions (e.g. isolates 8, 21385 and 2295). However, when isolates recovered from the experiment were sequenced although a *Phomopsis* was recovered it was different from the original inoculum (this was not the case for other isolates tested where the recovered isolate matched the inoculum). Therefore, it was necessary to repeat Koch's postulates for these three isolates, resulting in damage not being observed for isolates 8 and 21385 and significantly reduced for isolate 2295 (from 5/6 to 1/3).

Molecular characterization

The concatenated tree drawn upon both ITS and EF sequence data was successful in identifying all but one isolate obtained during the study. Isolates 8 and 9 belong to the *Phomopsis eres/conorum* species complex, isolates 2773 and 21386 were identified as *Phomopsis viticola* and isolate 21385 was identified as *Phomopsis theicola*. The known reference isolates of *Phomopsis vaccinii* previously obtained from Lithuania and the Netherlands were correctly identified, confirming the validity of this technique. Isolate 21389 belonged to an outlying group, sequence analysis was partially inhibited by the poor quality of the data for the elongation factor gene sequence (analysis is currently under further review). Using the concatenated tree in reference to previous published work with blueberry in Chile, this isolate belong to a cluster that contained species such as *Diaporthe phaseolorum* and *D. ambigua* but in practice based on the data to date cannot be accurately assigned to a species.

Table 7. *Phomopsis* characterisation

Isolate	Provenance	Molecular identity Combined ITS and EF sequencing	Growth rate (cm) (mean x,y)	Koch's postulates (x/y stems with symptoms)	Spore measurement (µm)			
					Alpha		Beta	
					Range Length	Range Width	Range Length	Range Width
8	Farm code H2R August 2010	*Phomopsis eres/conorum* complex	5.1 x 4.6	6/6* 0/3	2.5-10 6.2	2.5 2.5	17.5-30 22.3	1 1.12
9	Farm H2R . 'Duke' August 2010	*Phomopsis eres/conorum* complex	6.8 x 6.5	2/6				
2773	Farm E7X Ref. 21011972 June 2010	*Phomopsis viticola*	2.0 x 2.0	4/6	-	-	-	-
21385	Farm M10E cv. 'Chandler' R212/12	*Phomopsis theicola*	1.9 x 2.2	6/6* 0/3	5-12.5 6.95	1 1	17.3-35.0 23.9	1 1
21386	Farm M10E cv. 'Chandler' R213/12	*Phomopsis viticola*	6.7 x 6.5	4/6	-	-	-	-
21389	Farm M10E cv. 'Bluecrop' R216/12	*Phomopsis* sp.**		-	-	-	-	-
2295	*Vaccinium* ex Lithuania	*Phomopsis vaccinii*	4.6 x 4.4	5/6* 1/3	2.5-7.5 5.25	2.5 2.5	-	-
2360	Cranberry ex Netherlands	*Phomposis vaccinii*	1.5 x 1.5	6/6	-	-	-	-

*Koch's postulates repeated
**Identification work on this isolate is on-going.

Botryosphaeria

The three representative isolates were identified as belonging to two different species but from closely related genera - *Neofusicoccum australe (Botryosphaeria australis)* and *Botryosphaeria obtusa*. All isolates were found to be highly pathogenic with *Botryosphaeria obtusa* causing damage consistently. No underlying *Botryosphaeria* was identified in the un-inoculated controls.

Table 8. *Botryosphaeria* characterisation

Isolate	Provenance	Molecular identity (ITS)	Koch's postulates (x/y stems with symptoms)
16776	R198/12	*Botryosphaeria obtusa*	3/3
16778	R197/12	*Noefusicoccum australe*	2/3
21384	Blueberry Farm code B9H R221/12	*Noefusicoccum australe*	2/3

Discussion

Field symptoms

The various tip die-back symptoms observed and apparently caused by a range of fungi that include *Botrytis* and *Phomopsis* are notably less obvious in vigorous and carefully pruned plantations. Where the authors have visited plantations in other countries, they have observed less of this type of symptom.

Figure 9. An example of tip die-back.

The diversity of fungi found in the samples matched that observed by workers in other countries except in the absence of *Godronia cassandrae* (*Fusicoccum putrefaciens*). The incidence of *Botryosphaeria* was also relatively low.

Botrytis and *Fusarium* are frequent inhabitants of diseased wood but neither is thought likely to have caused the severe dieback responsible for dead and dying bushes in Herefordshire.

Descriptions of *Phomopsis* symptoms and disease development provided by researchers from both Michigan State University and North Carolina State University do fit much of what was observed in badly diseased British plantations. However these descriptions are recorded for *Phomopsis vaccinii*, a species that was not found in any samples tested as part of this project. Fera found evidence of more than one *Phomopsis* species in our samples. We are aware that several different species of *Phomopsis* have now been found associated with blueberry disease in North and South America.

Table 9. Die-Up or dieback?

Tip infection	Lesion	Hyphae grow or other propagule move downwards	**Die-back** of tissue starting at tip and developing downwards
Side infection	Lesion	Girdling of stem by fungal decay or hypersensitive reactions in local tissues	Tissues above the lesion wilt and die due to starvation (**Die-up**)
		Hyphae grow, or other propagule move, downwards	Tissue above the infection point may die if original lesion girdles the stem (**Die-up**)
			Lesions may develop further down the stem or in the crown where opportunities arise (mechanical, chemical, freeze injury?) causing **dead arm** like symptoms
Basal infection	Lesion	Hyphae grow, or other propagule move, upwards, killing tissue	**Dead arm** like symptoms
		Hyphae kill a large enough volume of tissue at the base of shoots or cause a hypersensitive reaction (tyloses etc.)	Tissues above the lesion wilt and die due to starvation (**Die-up**)

Table 8 describes ways by which observed symptoms may develop. We do not know whether basal infections result from the systemic movement of propagules within plant tissues or infection by spores from an external source. Such information is of critical importance for the design of control strategies and the prognosis for plantations already showing significant dead arm like symptoms.

At farm H2R symptoms developed rapidly and were observed in more than one field. There were patches within which many plants were worse hit than elsewhere in the field. Plants that appeared to suffer a sudden onset of disease tended to show dieback of the type shown in Figure 10 below and Appendix 1 (Figure 18). All varieties have been affected by the antler type symptom and/or dead arm but there appear to be significant differences in the incidence of these two symptom types between varieties. The dead-arm type symptom resulting being the most feared.

Figure 10. A shoot or cane dieback symptom (brown, necrotic rind)

Figure 11. Die-back of shoots. This preceded more severe symptoms expressed the following spring (H2R)

Table 10. Observed symptoms in commonly grown blueberry varieties.

Blossom blight	**Tip die-back /antlers**	**Dead-arm/Basal infection**
Cultivar most affected	**Cultivar most affected**	**Cultivar most affected**
Darrow	Darrow	Duke
Chandler	Chandler	Darrow
Draper	Draper	Draper
Aurora	Aurora	Legacy
	Duke	Bluecrop
		<u>Intermediate</u>
		Liberty
		Aurora

Literature Review (Year 1)

FAST looked at several scientific papers reporting the results of research into blueberry dieback, in other countries. Five papers that seem especially relevant to our project are briefly discussed here.

Studies in Michigan on stem canker development by *P. vaccinii* indicate that an abrasion wound or freezing damage is necessary for infection to occur. Although this occurred in North Carolina, it was not the primary means by which blueberry twig blight developed (Milholland, 1982). Milholland also reported that greenhouse inoculations had shown that systemic infection via the leaf was also possible. The same author undertook spore trapping, finding that conidia were collected from rainwater runoff from late February to early August (peaking in the period between blossom bud break and bloom). He reported that research in Michigan had shown a peak in the period from bloom to petal fall. Daykin & Milholland (1990) report pycnidia in necrotic stem tissues 10 weeks after inoculation of flowers. Hyphae had grown from the diseased flowers into the stem tissues.

In an earlier paper, Parker (1977) reported from Michigan that populations of *P. vaccinii* spores declined after peaking during bloom-petal fall and that no conidia were detected in traps after September.

Szmagara (2009) reported on a 3 year study of the biodiversity of fungi inhabiting blueberry plantations that: “The mycological analysis of fungi inhabiting stems [gave] 5,553 isolates belonging to 32 species”. A bar chart records the following split between fungal groups: 9.08% *Phoma* spp., 7.44% *Botrytis cinerea*, 7.38% *Topospora myrtilli* (syn. *Godronia cassandrae*), 4.56% *Phomopsis archeri*, 4.85% *Cytospora* spp. and 66.69% ‘other fungi’ of which the majority were common airborne moulds (*Alternaria, Epiccocum* and *Penicillium) or Fusarium* spp.

Szmagara also included an excellent photograph entitled “Necrosis and cracks of the bark of highbush blueberry stems and pycnidia of *Phomopsis* sp. and *Phoma* sp”. Perhaps the correct description should have been “pycnidia of *Phomopsis/Phoma”* and the author had been unable to distinguish whether either or both were present. Pycnidia of this type are easily found on similarly diseased wood at the Herefordshire and Devonshire sample sites. Szmagara wrote that *Phomopsis archeri* was most often isolated from stems of ‘Darrow’ and ‘Berkeley’ cultivars.

It was notable that Szmagara found a particularly high incidence of *Topospora* (syn. *Godronia*/*Fusicoccum*), a severe pathogen of blueberries that appears to have been absent from samples examined in our project.

Weingartner & Klos (1974) carried detailed work on the causes of stem dieback in Michigan. Under the sub-title "RESULTS – Association of fungi with symptoms and pathogenicity tests" the authors reported that fungi isolated from 3,130 segments of diseased blueberry tissue collected from 57 blueberry fields included *Godronia cassandrae* (40%), *Phomopsis vaccinii* (27%), *Coryneum microstictum* (3%) and *Fusarium* sp. (2%). However inoculations in the greenhouse showed that only *G. cassandrae* and *P. vaccinii* were pathogenic on actively growing 1 – 3 year old plants. It was also reported that, by comparison with *G.cassandrae*, wilting of otherwise symptomless stems was more common with *P. Vaccinii* infection. The average age of stems killed by *P. vaccinii* was 3.0 years. The fungus tended to grow downward through the stem, eventually killing major branches and often entire plants. Some infections were found in plant crowns, resulting in death of stems originating from the crown.

Literature review and liaison with overseas research teams (Year 2)

In Year 2 particular attention was paid to work carried out by two overseas groups: one led by Bernard Latorre at the Catholic University in Santiago, Chile and the other led by Annemiek Schilder at Michigan State University, USA.

An HDC travel bursary was used to visit Dr Schilder's laboratory at Lansing where a conference call between members of the SF 132 team, Dr Schilder and her graduate student, Casey Clemens.

Schilder and Clemens were about to publish the results of their own survey work on the causes of blueberry dieback. In marked contrast to the findings of the Latorre group and our own, the American survey found that the great majority of symptoms that were associated with *Phomopsis* were caused by *P. vaccinii*. Whereas *P. viticola* for example, is frequently isolated from diseased grapevines in Michigan it was not found to be an important component in blueberry disease there.

Wide experience of quarantine testing blueberry plants imported into the UK has been gained by Fera and it is known that *P. vaccinii* remains almost absent from the country.

Schilder and Clemens have also investigated several factors thought to increase the incidence of *Phomopsis* stem canker. Physical damage caused by winter frost and

mechanical harvest operations were found to be important factors. Bushes showing one or two dead canes due to *P. vaccinii* tend not to recover in subsequent seasons. Instead the bushes become progressively weaker, exhibiting more dead canes with time.

The Chilean team has published research into the range of fungi found to be associated with stem dieback of blueberries in Chile and published a paper in August 2013 entitled "Characterisation of *Diaporthe australafricana* and *Diaporthe* spp. associated with stem canker of blueberry in Chile". The study reported that at least four species of *Diaporthe* (syn. *Phomopsis*) are primary pathogens, capable of causing stem canker symptoms on blueberry in Chile. The authors noted that their paper was the first report of *D. ambigua*, *D. neotheicola*, and *D. passiflorae* attacking blueberries in that country.

Year 2 – Laboratory work

Phomopsis characterisation

Morphological characterisation based on the limited number of isolates available did not help to discriminate species or give any indication of potential pathogenicity (for example there was no relationship between faster growing isolates and greater incidence of damage). This is not surprising as morphological parameters alone are not considered reliable for species identification.

The combination of sequencing both ITS and EF genes and the use of a concatenated tree was successful in the majority of occasions in identifying *Phomopsis* isolates to species when sufficient reliable sequence data for the two genes was available on Genbank. ITS sequence data is prevalent as it has been used for many years to assist fungal taxonomy but based on previous experience ITS data alone cannot be used within the *Phomopsis* genus. Investigation of other genes for speciation for taxonomically challenging genera is becoming more common but there is still a paucity of information for reference isolates, resulting in some uncertainty. Therefore, the concatenated approach is a significant improvement in helping to identify *Phomopsis* to species but, as this work demonstrated, the lack of sequence data, especially for the elongation factor gene, can inhibit identifying more unusual species. Isolates obtained during this project showed considerable diversity and included *Phomopsis viticola* (2/6), *Phomopsis eres/conorum* (2/6), *Phomopsis theicola* (1/6) and an out-lying, and as of yet unidentified, *Phomopsis* sp. (1/6).

Data from the Koch's postulates studies initially suggested that most isolates could cause damage, however, interestingly, damage by isolates 8, 9 and 21385 was not actually caused

by the original inoculum but by another isolate of *Phomopsis*. Re-examination of the experimental procedure confirmed that this could not have been by cross contamination but due to the presence of the *Phomopsis* in the visually healthy material used for the study. When repeated, isolates previously thought to be pathogenic were predominately not able to cause damage. In summary, although based on a limited number of isolates, *Phomopsis viticola* was found to satisfy Koch's postulates and therefore have the potential to be the most damaging. *Phomopsis theicola* did not satisfy Koch's postulates and therefore may not be a primary incitant. The role of isolates from *Phomopsis eres/conorum* complex is less clear but would appear to be less damaging than *Phomopsis viticola*.

Our project isolated at least three different species of *Phomopsis* from symptomatic blueberries, based on molecular concatenated analysis. Of these *Phomopsis viticola* appeared to be the most damaging whilst the single isolate of *Phomopsis theicola* tested did not satisfy Koch's postulates and should not be considered as a primary pathogen. Of greatest interest was the isolation of *Phomopsis* from healthy looking commercially available young plants. This indicates that *Phomopsis* may be present in planting material but not causing any visual symptoms. This phenomenon may be attributed either to a latent infection, where there is a delay between infection and damage, and/or could indicate the presence of an endophytic phase which is also recognised as a problem in *Phomopsis* in fruit bearing crops. This could indicate that *Phomopsis* can live harmlessly within blueberry until some form of stimulus (e.g. physical, chemical, environmental, other biotic factors such as pests and diseases, senescence) stimulates the organism to cause damage.

Botryosphaeria characterisation

Botryosphaeria and *Neofusicoccum* are two genera previously isolated from blueberries with stem canker, but interestingly were not recovered from highbush blueberry with stem blight in Florida (Wright et al. 2010) but were recovered from blueberries in New Zealand (Sammonds et al. 2009) and from Chile (Espinoza et al., 2009). In the Chilean study *N. australis* was infrequently isolated in comparison to other species from these genera and was much less damaging in pathogenicity tests in comparison with *N. parvum.*

Our project recovered two species of *Botryosphaeria*ceae from symptomatic tissue that were consistently pathogenic to detached one-year-old stems. These isolates were consistently more pathogenic when compared to the *Phomopsis* isolates, indicating that *Botryosphaeria* may be of great significance. However, based on published research these organisms frequently coexist together in a disease complex. Interestingly, there was also was no

evidence of either latent or endophytic *Botryosphaeria* colonisation of the planting materials supplied.

Some months after the experimental work was completed, three of the 20 stock plants that had been grown in isolation at Fera were found to have died. *Phomopsis* was isolated from one and *Coniothyrium* from another.

The project has shown that progressive die-back of blueberries can be caused by more than one species of fungus, including those from *Phomopsis/Diaporthe*, *Botryosphaeria, Neofusicoccum* and *Coniothyrium*. At least some of these species may be able to live within blueberry shoot tissue without causing harm. Even if not already living inside blueberry tissues these species are common inhabitants of the plantation habitat, variously growing on such sites as old tip and bud infections, decaying prunings and injured flowers. When inoculated directly into wounded tissues the isolates of *Phomopsis* and *Neofusicoccum* collected from sick bushes show only moderate or even weak pathogenicity in laboratory conditions. However, it is also clear from field observations that crown/basal infections may extend sufficiently to cause wilting and starvation of canes. There is an increasing number of studies showing that *Phomopsis/Diaporthe* and *Botryosphaeriaceae* can survive within plants without causing disease but may then cause disease when their host becomes stressed by other factors.

For example, Dakin *et al* (2010) describe the development of severe dieback caused by *Neofusicoccum* disease in W. Australian peppermint plantations, proposing that their data: “support the hypothesis that [the disease] is caused by a common fungal endophyte, which is capable of causing disease in a stressed host. The disease is not caused by an introduced pathogen, The inciting factors leading to the decline are still unknown”.

While Dakin concluded that the dieback observed in peppermint “is not caused by an introduced pathogen”, the *Phomopsis* isolated from at least one of the farms surveyed for our project was not found to be present in dying plants tested in the spring of their first growing season after delivery. It was only in later samples that *Phomopsis* was found, perhaps ‘taking advantage’ of already ailing plants.

It is not known whether it is practically possible to eradicate these fungi from propagation sites, mother stock or cropping plantations nor whether eradication would make plants more or less susceptible to later disease infections.

Conclusions

In visits to blueberry plantations (Year 1), four types of symptoms were identified:

(1) Tip dieback;

(2) Tip dieback associated with dead flower;

(3) Progressive tip dieback

(4) Die-up with crown death.

- Symptom (4) was most associated with blueberry death.
- Symptom (1) Tip dieback was present in all sites visited.
- Symptom (4) Die-up was clearly present in around half the sites visited.
- In Year 1, a range of fungi was isolated from the various symptom bearing samples, including *Phomopsis, Phoma, Fusarium*, *Cytospora, Coniothyrium, Botrytis* and *Botryosphaeria*.
- No consistent fungus was isolated from the various symptoms. *Phomopsis* was the most commonly isolated. Several different fungi cause similar dieback symptoms.
- At least three different species of *Phomopsis* were isolated from symptomatic blueberries based on molecular concatenated analysis (*P. viticola*, *P. eres/conorum* and *P. theicola*).
- Of these *Phomopsis viticola* appeared to be the most damaging. A single isolate of *Phomopsis theicola* tested did not satisfy Koch's postulates and should not be considered a primary pathogen without further investigation. However a similar, weakly pathogenic, result was obtained with a Lithuanian isolate of *Phomopsis vaccinii,* a species known to be highly pathogenic to blueberries.
- Of great interest was the isolation of *Phomopsis* from healthy looking commercially available young plants. This indicates that *Phomopsis* may be present in planting material without causing visual symptoms. This phenomenon may be attributed either to a latent infection, where there is a delay between infection and damage, and/or could indicate the presence of an endophytic phase, which is also recognised as a problem in *Phomopsis* in other fruit bearing crops.
- This could indicate that *Phomopsis* can live harmlessly within blueberry until some form of stimulus (e.g. physical, chemical, environmental, other biotic factors such as pests and diseases, senescence) stimulates the organism to cause damage. This conclusion is supported by observations made by pathologists work on similar diseases affecting other crops, including those caused by *Botryosphaeriaceae*.
- Two species of *Botryosphaeria*ceae were recovered from symptomatic tissue and were consistently pathogenic to detached one-year-old stems. These isolates were consistently more pathogenic when compared to the *Phomopsis* isolates, indicating that *Botryosphaeria* may be of great significance. However, based on published research

these organisms frequently coexist together in a disease complex. Interestingly, there was also was no evidence of either latent or endophytic *Botryosphaeria* colonisation of the planting materials supplied.

- In-field symptoms do not provide a reliable indication of the presence of particular species of *Phomopsis* or *Botryosphaeriaceae* nor whether species from these genera are the first cause of die-back symptoms. However it is clear from work carried out for this project that there is a need to protect bushes from these species. The presence of these fungi associated with tip dieback symptoms and their known ability to persist on detached, decaying plant material should serve as a reminder to growers that plantation hygiene is of critical importance and that there are range of points for these pathogens to enter blueberry tissues.
- UK Field experience has been that bushes exhibiting die-up symptoms tend not to recover, becoming progressively weaker over a period of years. This experience matches that of American researchers monitoring bushes infected by *P vaccinii*.
- The speed with which bushes succumb to serious dieback symptoms would appear to be controlled by factors other than the simple pathogenicity of the causal fungus. The fact that pathogenic *Phomopsis*, *Botryosphaeraceae* and other fungi are found in dead tissues associated with dieback that progresses slowly, or not at all for an indefinite period but at other times is clearly playing a role in the relatively sudden and complete death of canes or whole plants, would suggest that other aspects of plant health play an important role in limiting disease development. Blueberry crowns are often a complex mixture of healthy shoots and older, decaying wood. In weaker plants the border between healthy and decaying tissue is not stable.

Knowledge and Technology Transfer

11 February 2013	The collaborators attended and contributed to the Stem Diseases Workshop held at Fera, York
18 July 2013	Charles Lane included results from the project in a presentation entitled “Diaporthe (Phomopsis) dieback of berry fruit” given to growers and researchers attending “Fruit For the Future” event at the James Hutton Institute, Dundee.
21 November 2013	Graham Moore presented a summary of Yr 1 results to the EMRA/HDC Soft Fruit Day at East Malling Research, Kent. Information from the presentation was used in articles published in both the HDC News and the Fruit Grower magazine.
20 February 2014	The HDC included an item describing the project and interim results in the Soft Fruit Review 2013/2014 magazine.

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Glossary

Abiotic factors	Physical rather than biological; not derived from living organisms
Biotic	Produced or caused by living organisms
Phomopsis	Asexual state of *Diaporthe*
Diaporthe	Sexual state of *Phomopsis*
Neofusicoccum	Previously described as *Botryosphaeria.* Genus is a member of the *Botryosphaeraceae*
Phomopsis / Phoma	Due to similar appearance of symptoms and one spore type these species are difficult to distinguish without reference to biochemical techniques

Appendix 1

Throughout the period of the project work, the team photographed field symptoms before submitting samples to Fera for diagnostic work. On receipt and during sub-sampling, Ann Barnes and her team also took photographs. Some examples are shown below. It is difficult and in many cases, impossible, to distinguish between symptoms caused by several different species.

Tip and flower symptoms

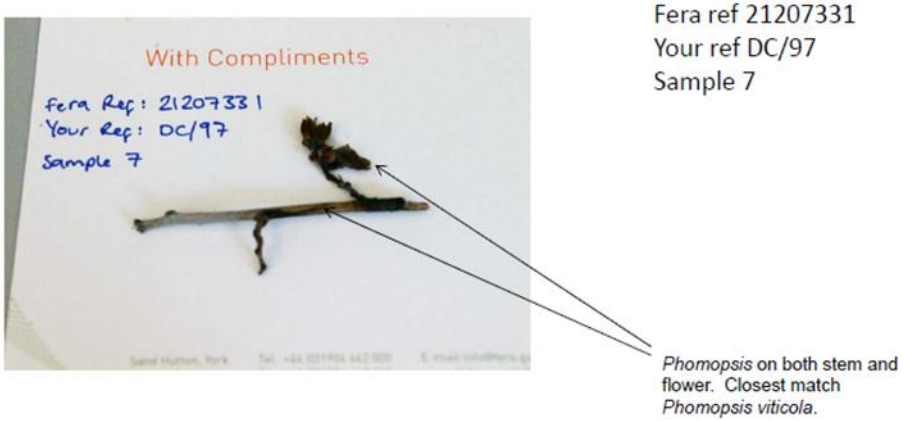
Phomopsis and *Botrytis* would seem to be important causes of blossom blight but tests on decaying flowers also yielded other fungi, including *Ascochyta* and *Sematosporium.* However relatively few flowers samples were submitted for testing. Dead material from twigs, often associated with sick blossom clusters of spent flower strigs (peduncles) perhaps yielded the widest variety of fungi. *Phomopsis* was more likely to be isolated from the proximal edge dead shoots than from the distal part, perhaps indicating that the distal parts had failed due to starvation and not the direct effect of infection. Some of the most dramatic symptoms, such as those shown in Figure 18 (*Phoma*) did not yield species that are thought to play an important role in the development of death of bushes.

Crown infections

Phomopsis, *Neofusicoccum* and *Coniothyrium* were all found to be associated with similar wood rotting symptoms (Figures 13, 14 and 16) and it seems clear that field diagnosis based on dissection of crowns would not provide a safe conclusion. Given that an expanding lesion ultimately cuts off water and nutrients to distal parts of an affected bush, all have the potential to cause deficiency and 'dead arm' type symptoms. Death of shoots would tend to progress from tip backwards but the symptoms on these parts would be due to starvation or perhaps colonisation by other fungi. *Fusarium* was also a common inhabitant of diseased crowns (Figure 17) but, based on experience the team concluded that this fungus was not responsible for initiating disease.

Sample as photographed in the field, Herefordshire

cv. ‘ Draper’



Sample as photographed at Fera, York

Figure 12. *Phomopsis viticola* associated with a form of tip dieback

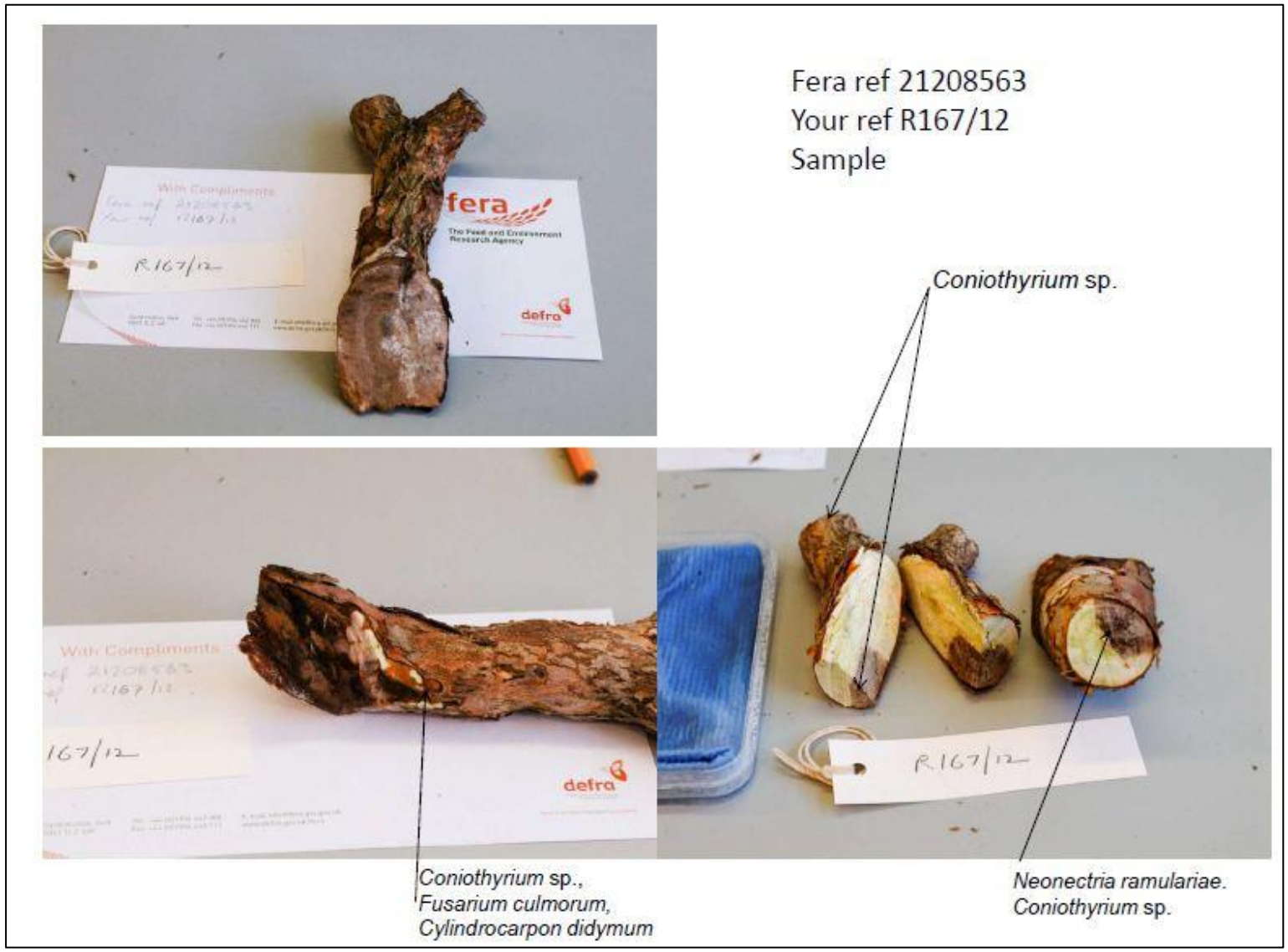


Figure 13. *Coniothyrium* associated with a damaging 'wood rot' type basal lesion. Three other fungi also isolated from same sample.

Figure 14. Crown rot from which *Phomopsis* was isolated but note similarities with symptoms shown in Figure 13

Figure 15. Basal wood rot from which either *Fusarium* species or no primary pathogens were isolated.

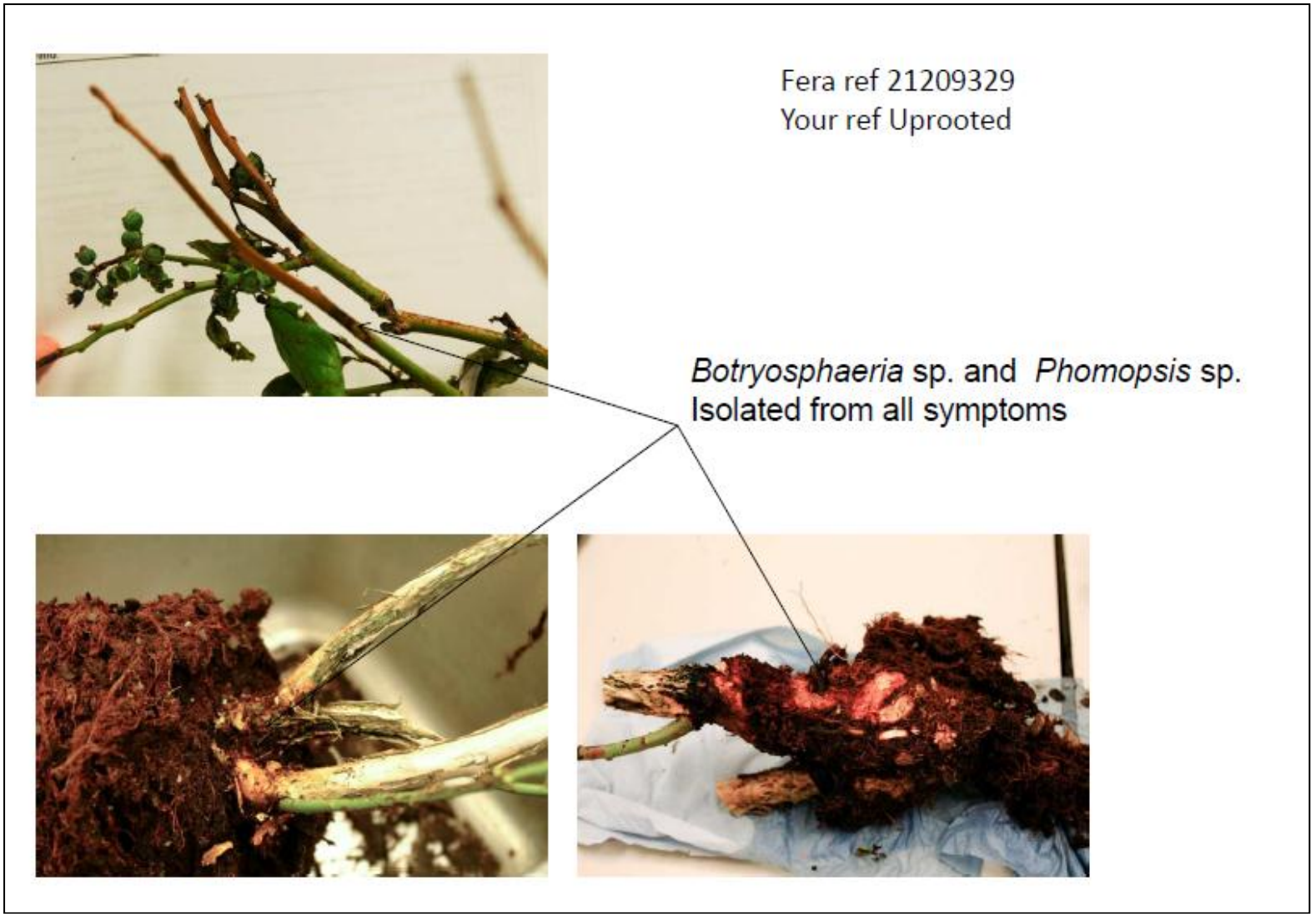


Figure 16. Sick bush found to exhibit crown and shoot tip symptoms. *Botryosphaeria* and *Phomopsis* isolated from all symptoms.

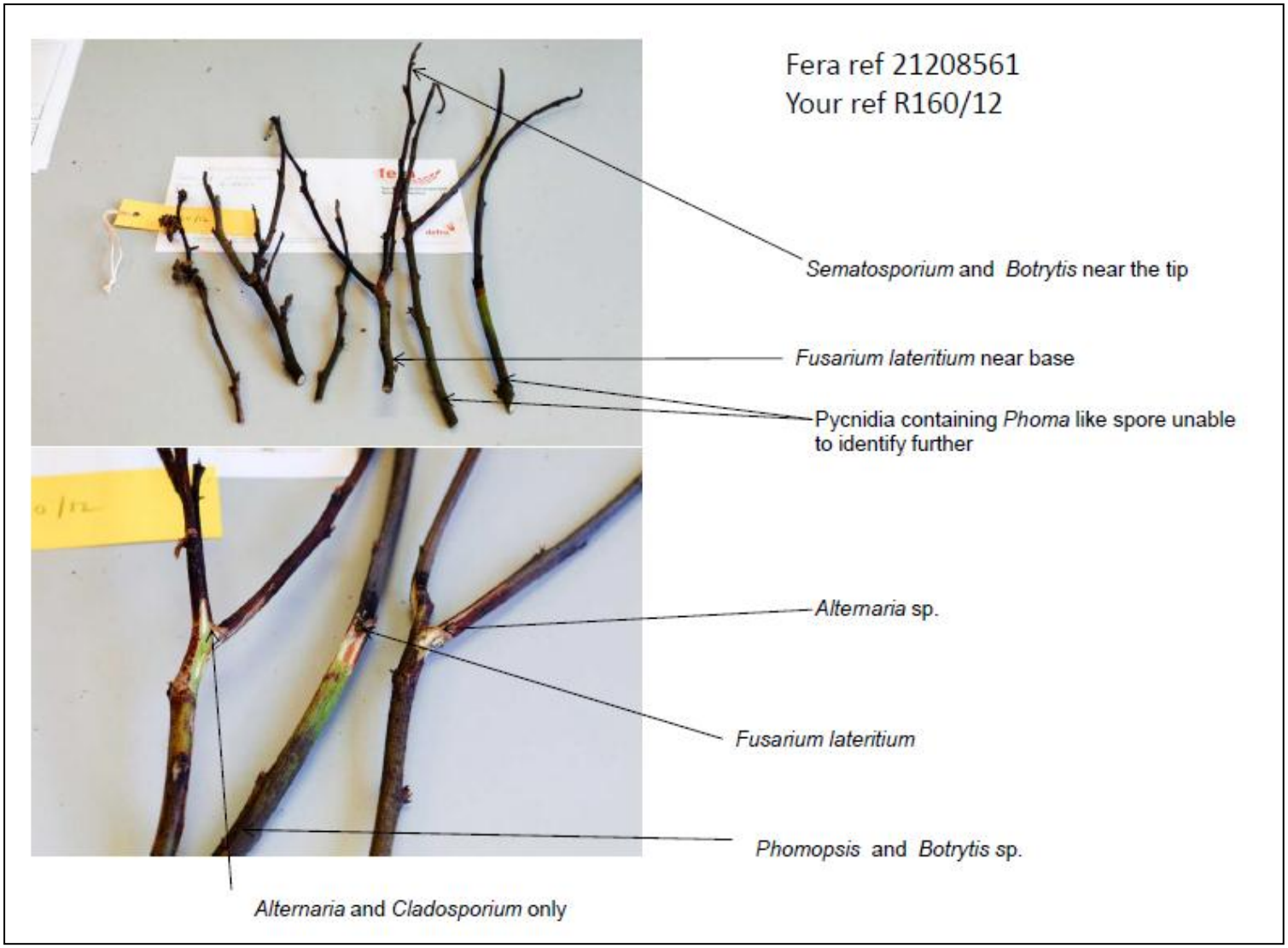


Figure 17. Shoots with severe dieback. Note that several species of fungi were isolated from affected tissue.

Fera ref 21207325
Your ref DC/97
Sample 1

Phoma

cv. ‘Duke’

Figure 18. Striking dead arm symptom, with brown rind, on cv. 'Duke'. This symptom was characteristic of the sick bushes at one of the more severely affected sites

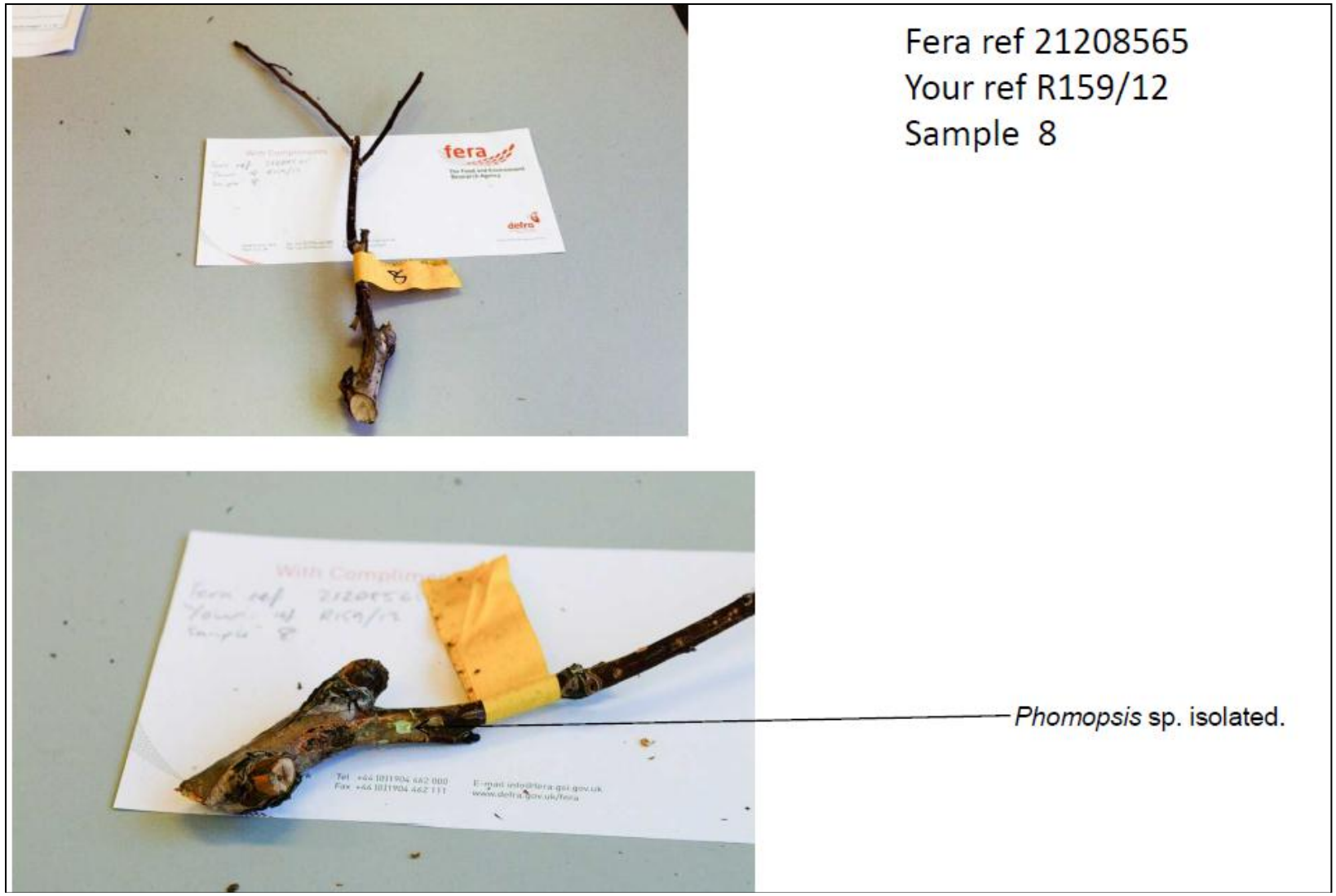


Figure 19. *Phomopsis* isolated from the proximal end of an infected shoot.

Appendix 2

Photographs charting the progress of tip dieback at farm H4R during the summer of 2013.

Figure 20. Tip dieback associated with bud infection (21 March 2013)

Figure 21. 16 April 2013

Figure 22. 30 April 2013

Figure 23. 14 May 2013

Figure 24. 29 May 2013

Figure 25. 25 June 2013

Figure 26. 9 July 2013

Figure 27. 23 July 2013

Figure 28. 2 September 2013

Figure 29. 24 October 2013

Appendix 3

Blueberry site visits by EMR in 2013

Method

Blueberry plantations identified during visits in 2013 as having die-back and or die-up problems were visited in 2013 to check on the progress of the problem. Three sites were visited: H4R, C1O and M11E.

Results

H4R

The site was visited on 31 July. Only the field pots on LHS at the bottom of the hill from the farm were looked at. In 2012 there were many pots affected with tip die-back and bush death. The first block examined (cv. Duke?), which was the worst in 2012, had improved considerably. Most plants looked healthy with very little tip die-back evident. However, some bushes still showed symptoms of the die-up and a number of bushes were dead (approx. >20 pots in first few rows). Some samples of the affected bushes were taken. In the adjacent block (Bluecrop?) the incidence of tip die-back seen in 2012 had dropped considerably. Some tip die-back was observed, associated with a dead flower.

M11E

The site was visited on 1 November 2013.

Established plantation of mixed cultivars - The main problem in this plantation seen in 2012 was crown death. The problem here has continued to progress with many gaps now present in the plantation. Some samples weretaken.

New plantation cv. Liberty - This was planted in 2012. Very few plant deaths were seen in 2012 but a low incidence of shoot die-back was present. The grower said that in 2013 around 100 dead bushes had been removed from the new planting. Of the remaining plants about 1% showed dieback and weak growth. These were examined and many of these had die-back resulting from the failure of the root ball to develop a new root system. Some grubbed bushes left in the alleyways showed symptoms of atrophied stem bases, possibly as a result of insect damage. Two affected plants were sampled.

C1O

The sites were visited on 25 October 2013.

Site 1 Honey Tye - This was an established plantation which showed a high incidence of die back in 2012. In 2013 the site had improved with better bush growth and very little die-back

Site 2 New planting tunnelled (Mainly cv. Duke)- This was not visited in 2012. The site was planted out in 2011 from potted blueberries. The field site was on raised beds through Mypex and under tunnels. The grower reported that the bushes grew well in 2012 but then in 2013 showed bush death or partial death, symptoms typical of the problem under investigation. Some bushes were dug up and the roots in general were okay but the crown was dead, with obvious progressive dieback. Several of the dead ones also showed very little root development and failure to root out from the root ball, similar to M11E site. The grower commented that in 2012 in the autumn conditions for growth remained favourable for some time, making the shoots very vulnerable to frost damage. Secondary fungi colonising the frost damage may have contributed to the dieback.

Isolations from samples

Isolations were made from the various crown rots and die-backs. In all cases *Phomopsis* sp. was the predominant or only fungus recovered.

Discussion

The improvement of bush health in several of the sites suggests that growing conditions in 2012/2013 may have been very favourable for bush growth so that the bushes out grew the infections. This supports the idea that the blueberry death issue may be a complex interaction between fungi already in the plant and the growth of the plant, rather than one particular disease problem. Certainly there is evidence for *Botryosphaeria* existing as an endophyte in apple trees and conditions such as stress, cold winters and fungicide use determining whether the fungus causes disease symptoms on the tree. In this respect it may be worth evaluating alternative chemicals, in particular plant strengtheners, elicitors and growth promoter type products for their effect on disease expression. Also methods to control *Phomopsis* etc in the nursery and a test to check nursery material to ensure freedom from certain fungi may also be appropriate.