Project title:	Branch dieback in blackcurrant: identification and control of potential pathogens, including the fungus *Phomopsis.*
Project number:	SF 012 (GSK223)
Project leader:	John Scrace, Food and Environment Research Agency (Fera), Sand Hutton, York, YO41 1LZ
Report:	Final report, May 2011
Previous report:	None
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Location of project:	Fera, Sand Hutton, York
Industry Representative:	Rob Saunders, GlaxoSmithKline
Date project commenced:	May 2010
Date project completed (or expected completion date):	May 2011

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

I declare that this work was done under my 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

Diaporthe strumella (also known as *Phomopsis ribicola*) has been recovered consistently from plants suffering from the severe branch dieback problem currently affecting some blackcurrant varieties. In host inoculation tests it has been shown to be a primary pathogen and to reproduce some of the symptoms of the problem.

Background and expected deliverables

Branch dieback in commercial blackcurrant plantations, particularly of the varieties Ben Avon and Ben Tirran, has been a significant problem in the last three years. The severity of the problem and its speed of spread through the plantation have varied between sites, but in the worst affected plantation 80% of plants became affected within the space of one year, leading to very significant yield loss.

In 2009, samples of affected Ben Avon plants from this plantation were sent to the Plant Clinic at Fera. The main symptoms seen in the affected branches were decay of the wood and pith at the base. Root rotting was also noted.

Tests on the sample resulted in the recovery from the branches of a species of the fungus *Phomopsis*. Fungi from this genus are found commonly in association with branch dieback on a range of woody plants, but whilst some are aggressive primary causes of disease others are regarded as weak pathogens, colonising plants already stressed, damaged or dying back due to other factors (including infection by other diseases).

It was not known at this time whether the precise symptoms seen in this first sample were present at other affected sites, or whether the problem differed between plantations. It was also not known if the *Phomopsis* recovered from the sample was the primary cause of the problem, if other pathogens might be involved, or whether the cause itself also varied between affected sites.

The aim of this project was therefore to gain an improved understanding into the cause or causes of blackcurrant dieback, including the precise symptoms of the problem and the role played in it by *Phomopsis* and any other potential pathogens. This would be done by visiting affected sites and examining samples of affected plants. The symptoms would be recorded and tests carried out to see which potential pathogens were recovered with consistency

from the plants. Any candidate pathogens would be inoculated into healthy plants to see if they reproduced the symptoms of the problem.

Whilst no symptoms of the dieback problem had been seen on stoolbed plants at the time the project started, visits would also be made to stoolbeds (once the stems to be harvested for cuttings had matured) to check for evidence of pathogens.

Preliminary fungicide screens (using laboratory-based *in vitro* methods) would also be carried out against any potential pathogen recovered.

Summary of the project and main conclusions

Visits to plantations affected by the branch dieback problem, and subsequent laboratory examination, revealed that the symptoms of the problem were consistent between sites. The symptoms seen most commonly are:

External Symptoms

In spring / early summer:

- Failure of some branches to leaf out
- Production of leaves of reduced size on some branches
- One or both of these symptoms may be present on a bush, often together with healthy-looking branches
- In extreme cases all of the branches on an affected plant may fail to produce leaves

As summer progresses, the above symptoms may still be seen, but additionally:

- Wilting of leaves may develop on some branches
- Affected leaves turn yellow and then brown, and dead leaves remain attached to the branches for a considerable time
- The wilting symptom may develop on branches that had produced leaves of reduced size in spring, or on branches that had previously appeared healthy

Tiny, black fungal fruiting bodies (visible with a hand lens) may occasionally be found, particularly if a branch has been dead for some time. They are most often found in the lenticels (air pores).

Internal Symptoms

- A firm, brown decay of the wood within affected branches, particularly at the base, with an associated brown decay of the pith
- The decay of the pith often extends beyond the limits of the wood decay
- Fungal mycelium (usually only visible with a low-power microscope) is present within the decayed pith
- Parts of the crown may be affected by a brown decay, usually closely linked to the affected branches
- The tops of some of the main roots, immediately below affected parts of the crown, may also be decayed

In laboratory tests, a *Phomopsis/Diaporthe* species (these are two different stages of the same fungus) was recovered consistently from affected branches. It was also sometimes found within the rotting crowns and roots. No other potential pathogens were found with any consistency.

Identification of the species of *Phomopsis/Diaporthe* proved difficult. Sequencing of the fungal DNA did not provide a precise species identification, but showed that the same unknown species was present at all of the plantation sites visited. From the morphology (appearance) of the various fruiting body and spore types produced by the fungus, it was finally identified as *Diaporthe strumella* (also known as *Phomopsis ribicola*). This has been found previously on dead blackcurrant branches, but there was no information as to whether it was likely to be the primary cause of the dieback.

In tests in which the *D. strumella* has been inoculated into the branches of healthy, potted blackcurrant plants, and also into healthy detached branch sections, it has been possible to reproduce the internal symptoms of wood and pith decay and to recover the fungus again from these symptoms. This confirms that *D. strumella* is capable of causing the symptoms found in affected plantations, and satisfies the requirements of a test called Koch's Postulates that proves the pathogenicity of a potential disease-causing organism. The inoculation work also suggested that strains of the fungus may exist, some of which could be more aggressive than others.

Visits were also made to stoolbed sites supplying cutting material for use in fruiting plantations. It was possible to find low levels of *D. strumella* at the majority of these sites. In some cases the stoolbed stems from which the fungus was recovered showed internal

symptoms of wood and pith decay identical to those found in the affected plantations, but in others the fungus was found on stems that had appeared healthy at the time of sampling.

Tests to determine the minimum, maximum and optimum temperatures for growth of *D. strumella* showed that the fungus is well-adapted to the conditions likely to be experienced in UK blackcurrant plantations.

Preliminary *in vitro* screening of fungicides showed that the products Switch (cyprodonil + fludioxonil), Bravo 500 (chlorothalonil), Signum (pyraclostrobin + boscalid) and Systhane (myclobutanil) may be worth evaluating in the field. These products are already used at some of the affected plantations in programmes to control other diseases of blackcurrant, but it is possible that a critical timing for the control of *D. strumella* is missed in these programmes.

At present the sources of the fungus, and the methods by which it spreads in plantations, are still poorly understood. Detailed further work is required into both the epidemiology and the control of the disease.

Financial benefits

It is not currently possible to give an accurate estimate of the financial benefits, given that the majority of the work carried out so far has been diagnostic work aimed at determining the cause of the problem. Only very limited work (preliminary *in vitro* screening of fungicides) has so far been conducted into the control of the problem.

During the course of this project close links have been made with researchers in Europe carrying out on work on diseases caused by *Phomopsis/Diaporthe.* This has already led to the possibility of developing a screening method for blackcurrant planting material, based on a serological test developed by researchers in Germany. It is likely that these links will result in other benefits, not just for this project but for other crops where problems are caused by *Phomopsis/Diaporthe* (e.g. blueberries, grapevines).

Action points for growers

Based on the findings in this project, and knowledge of diseases of other crops caused by *Phomopsis/Diaporthe* species, it is possible to give some general recommendations to reduce the likelihood of outbreaks of blackcurrant dieback, or to reduce the impact of an outbreak where it does occur:

- Recognise that *Diaporthe strumella* is a primary fungal pathogen capable of causing serious dieback.

- Aim to provide the best possible growing conditions so that plants do not come under stress. Avoid planting the varieties most prone to attack (Ben Avon and Ben Tirran) on all but the most suitable sites (for example, avoid sites prone to drought, waterlogging or desiccating winds).

- Avoid, where possible, physical damage to the bushes during cultural operations – this will reduce the number of wound sites through which *D. strumella* may be able to infect branches.

- If symptoms develop, remove branches affected by dieback, and clear up and dispose of all dead branch material at the bases of the plants. This should reduce the likelihood of production of fruiting bodies and spores by *D. strumella*.

SCIENCE SECTION

Introduction

Dieback of the branches of blackcurrant bushes has become a significant problem in the last three years. Whilst it is fairly rare for plants to be killed completely, a large percentage of branches on a bush can become affected. The varieties affected most commonly are Ben Avon and Ben Tirran, although symptoms are seen occasionally on other varieties such as Ben Alder and Ben Gairn.

The severity of the problem varies between plantations, but in one plantation 80% of plants have been affected, at an estimated cost of £5,000 per hectare per year for this plantation alone. Severe attacks have also led to the premature grubbing of plantations.

Samples from affected Ben Avon bushes were received at the Fera Plant Clinic, Sand Hutton, York in August 2009. Isolations from wood decay at the base of affected branches led to the recovery of the fungus *Phomopsis*. The roots immediately below the affected branches also showed symptoms of decay, but no evidence was found of *Armillaria* (honey fungus), *Phytophthora* or other root pathogens.

Phomopsis has been associated with dieback of blackcurrant previously (Berrie, undated), but there is little or no information available as to the species involved or the epidemiology of the problem. The fungus is also known as *Diaporthe.* *Phomopsis* is the asexual stage and *Diaporthe* the sexual stage. Both stages of the fungus can often be found on woody plants affected by *Phomopsis/Diaporthe* dieback diseases). From this point onwards, unless an individual species is mentioned, the fungus will be referred to as *Phomopsis/Diaporthe* (see Glossary for definitions of the types of fruiting body and spore produced by each stage of the fungus). Some *Phomopsis/Diaporthe* species are known to cause significant dieback problems in other crops (e.g. blueberry, pear), but there are also a large number of species that are regarded as weak pathogens, colonising plants that are weakened or dying back due to other factors (including infection by other diseases). For example, five different species of *Phomopsis/Diaporthe* have been isolated from dieback of blueberry and cranberry bushes, and these have varying degrees of significance.

The aim of this project was to gain an improved understanding into the cause or causes of blackcurrant dieback, including the precise symptoms of the problem and the role played in it by *Phomopsis/Diaporthe* and any other potential pathogens.

Materials and methods

1. Site visits

1.1 Fruiting plantations

Visits were made in spring and summer 2010 to affected plantations to view the distribution, symptoms and severity of the problem in the field and to take representative samples of affected plants (between two and five plants per site) for laboratory processing.

Table 1. Fruiting plantations visited

Site / sample reference	Variety	Date visited
1/9852	Ben Avon	13/5/10
1/9853	Ben Gairn	13/5/10
2/9854	Ben Avon	13/5/10
2/9855	Ben Tirran	13/5/10
3/9856	Ben Avon	13/5/10
3/16411	Ben Avon	N/A – additional sample sent to Fera by grower on 02/8/10
4/15816	Ben Tirran	26/7/10

1.2 Stoolbeds

In winter 2010 visits were made to stoolbed sites, from which hardwood cuttings are taken and supplied to growers for the establishment of fruiting plantations. Stems were sampled at random (one stem removed per plant at the base using secateurs) throughout the stoolbed and returned to the laboratory for testing.

Table 2. Stoolbed sites visited

Site / sample reference	Variety	Date visited	Number of plants in stoolbed	Number of stems sampled
5/27999	Ben Avon	7/12/10	43	20[A]
5/28000	Ben Avon	7/12/10	4,000	150[B]
5/28001	Ben Tirran	7/12/10	3,200	150[B]
6/28580	Ben Avon	14/12/10	1,000	100[C]
6/28581	Ben Tirran	14/12/10	1,000	100[C]

A: Sample size gives a 47% chance of detecting a single affected plant within the stoolbed.
B: Sample size gives a 95% confidence level of detecting a 2% level of infection.
C: Sample size gives a 95% confidence level of detecting a 3% level of infection.

2. Laboratory processing of samples

2.1 Fruiting plantations

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

2.1.1 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 two to three 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 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. See appendix 2 for agar recipes.

2.1.2 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.

2.1.3 Baiting techniques

Decayed crown and root tissues were tested for the presence of *Phytophthora* using a baiting technique. The plant tissues were immersed in Petri's solution (see appendix 2 for recipe) in sealed plastic containers, together with leaf sections of rhododendron (a reliable bait for many species of *Phytophthora*). After three to five days, the rhododendron leaf sections were washed twice with deionised water and placed onto PARPH agar. The agar plates were incubated for six days and then examined for growth of *Phytophthora*.

2.1.4 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.

2.2 Stoolbeds

Stems from the stoolbed sites were examined for any external or internal symptoms of decay. Any stems with symptoms were tested by a combination of aseptic isolation (onto ¼PDA + P&S) and humid incubation, as described above.

The remaining stems were tested for latent (symptomless) infection by humid incubation in clean seed trays containing damp paper towel. Twenty-five stems were placed into each seed tray. Each tray was then enclosed in a sealed plastic bag to produce high humidity, and incubated at ambient temperature. The incubation tests were assessed firstly after three weeks, and periodically thereafter.

3. 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).

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.

Identification of *Phomopsis/Diaporthe* species is currently complicated by the limited amount of molecular data available and also by the need for a complete revision of both molecular and morphological information. To obtain a consensus of opinion on the most likely species of *Phomopsis/Diaporthe* involved in this case, plant material and cultures were also sent for examination by Dr Paul Cannon of CABI Bioscience (Egham, Surrey), Dr Jutta Gabler of the Julius-Kühn Institute (Germany) and Dr Gerard van Leeuwen of the Netherlands Plant Protection Service, all of whom have considerable experience and expertise in identifying and working with this fungus.

4. Host inoculation tests

In order to determine whether a fungus isolated from a disease symptom affecting a plant is the actual cause of that symptom, a process known as Koch's Postulates must be completed. The fungus is inoculated into a healthy plant, where it must reproduce the symptoms of the disease. It must then be re-isolated from the plant.

Various techniques were used to inoculate plant material with potential pathogens recovered from the plantation material.

4.1 Inoculation of potted plants

The plants used for the inoculations were variety Ben Tirran growing in four litre pots, purchased from Bridgemere Nursery, Bridgemere, nr. Nantwich, Cheshire. Inoculations were made using pieces of colonies (mycelium only) growing on agar, taken from close to the advancing margin of seven-day old cultures of the test fungus on PDA. One plant was used for each fungal isolate tested and a control plant was inoculated with plain agar without the fungus. The plants were inoculated in a variety of ways (separate branches of the plant were used for each technique):

1. A wound was made by cutting into the bark of a woody branch with a sterile scalpel to a depth of approximately 2mm, creating a 'tongue' of bark approximately 1cm long, under which a block of agar from the culture (approximately 10 x 5mm) was placed, with the fungal mycelium in contact with the plant tissue. The cut was made in an area where a flower truss or side-shoot had just been removed, to provide a close link with the vascular tissues. The wound was wrapped in damp cotton wool, followed by one of the following: parafilm, clingfilm, or clingfilm and black plastic, in each case held in place by electrical insulation tape. The different wrapping techniques were intended to create varying degrees and persistence of humidity.
2. An agar block of similar size was placed with the mycelium in direct contact with unwounded bark of a woody branch and then wrapped with damp cotton wool, clingfilm and black plastic, held in place by electrical insulation tape.
3. An agar block of similar size was placed in direct contact with a current year's green shoot. A leaf was removed and the agar placed over the wound. The inoculated area was wrapped in damp cotton wool and clingfilm, held in place by electrical insulation tape.

The plants were placed on an outdoor standing area below shade netting and watered as required.

In a separate test a single plant was inoculated with spores of *Phomopsis/Diaporthe*. A wound was created as in technique one above, but inoculated with either a spore suspension containing 1600 x 10^4 α-spores of *Phomopsis*, or fruiting bodies (perithecia - obtained from incubated blackcurrant branches) containing ascospores of *Diaporthe* (see Glossary for a description of these spore types). The inoculated area was wrapped in damp cotton wool and clingfilm, held in place by electrical insulation tape.

4.2 Inoculation of detached branch material

Thirty centimetre lengths cut from healthy branches of Ben Avon were inoculated using pieces of colony (mycelium only) taken from close to the advancing margin of seven-day old cultures of the test fungus on PDA. The branches were first dipped in industrial methylated spirit (IMS), which was allowed to evaporate off (this was an attempt to remove any surface contaminants). They were then inoculated in different ways (two branch sections were used for each technique, plus a control branch inoculated with plain agar):

1. A wound was made by cutting into the bark with a sterile scalpel to a depth of approximately 2mm, creating a 'tongue' of bark approximately 1cm long under which a block of agar from the culture (approximately 10 x 5mm) was placed, with the fungal mycelium in contact with the plant tissue.
2. A wound was made by cutting out completely a section of bark of approximately 10 x 5 mm. A block of agar of the same size was then placed onto the wounded area (mycelium in contact with the plant tissue), after which that part of the branch was wrapped with Parafilm.
3. A small piece of pith (no more than 2-3mm) was removed at the base of the branch and replaced by a piece of culture of similar size. The basal 1cm of the branch was then wrapped with Parafilm.
4. An agar block (approximately 10 x 5mm) was placed directly onto unwounded bark, with the mycelium in contact with the plant tissue.

The branch sections were placed on damp filter paper in a clean seed tray, which was enclosed in a plastic bag to create high humidity and incubated at ambient temperature. The branches were examined after six weeks incubation.

In a later test, 30cm lengths of branch (Ben Tirran) were inoculated using technique 3 (3 branch sections per isolate, plus controls) but no damp filter paper was added to the seed tray in which the branch sections were incubated.

5 Tests to determine cardinal temperatures of Phomopsis/Diaporthe

Five millimetre agar plugs were taken from the advancing margin of PDA cultures of each of the test isolates, and placed into the centre of each of eight further PDA plates. Each of these plates was incubated at a different temperature, the range being 5, 10, 15, 20, 25, 30, 35 and 40°C. The diameter of the colonies was measured at two to three day intervals over the next 20 days to determine maximum, minimum and optimum temperatures for growth. There were three replicates per isolate.

6 *In vitro* tests of fungicide activity against *Phomopsis/Diaporthe*

Tests were carried out to assess the activity of candidate fungicides against mycelial growth and spore germination of *Phomopsis/Diaporthe*. The fungicides evaluated were Bravo 500 (chlorothalonil), Cercobin (thiophanate-methyl), Signum (pyraclostrobin + boscalid), Switch (cyprodonil + fludioxonil) and Systhane (myclobutanil).

6.1 Mycelial growth assay

Five millimetre agar plugs were taken from the advancing margin of PDA cultures of each of the test isolates, and placed into the centre of PDA plates amended with the candidate fungicides at the following rates: 0, 0.1, 1, 10 and 100ppm. Three replicates were used for each isolate. The plates were incubated at 20°C and the diameter of the colonies was measured after 5 days. Dose response curves were plotted and EC_{50} values calculated from these. The EC_{50} is defined as the fungicide concentration at which growth of the fungus is inhibited by 50% compared to its growth in the absence of the fungicide.

6.2 Spore germination assay

The effects of the candidate fungicides on the germination of *Phomopsis* α-spores was determined using a technique adapted from Pijls *et al* (1994), which uses optical densitometry to measure the amount of spore germination. The fungicides were tested at concentrations of 0.0001, 0.001, 0.1, 1, 10 and 100ppm.

Dilutions of each fungicide were prepared in a glucose-peptone growth medium (GPM) (see appendix 2 for recipe). 100µl of each test concentration was pipetted into wells in flat-bottomed microtitre plates (96 well), using three replicates for each concentration. 100µl of unamended GPM was used as the control concentration.

Spore suspensions were produced from spores obtained from pycnidia of *Phomopsis* produced on cultures on PDA or 1/4PDA + P&S. Spores were harvested in 5ml of GPM. Spores were counted and the concentration used in the test adjusted to 10,000 spores/ml.

150µl of the spore suspension was added into each treatment well and 150µl of GPM was pipette into control wells.

Absorbances were read across all wells on the plates at 450nm immediately after the addition of spores and then after six day's incubation at 20°C. The absorbance readings were used to calculate the % inhibition compared to the control. Dose response curves were plotted and EC_{50} values determined.

Results

1. Site visits

1.1 Fruiting plantations

Symptoms of the dieback problem were very similar at all of the plantations visited, although there were some differences between the visits carried out in May and those conducted in July.

At the sites visited in May (1/9852, 1/9853, 2/9854, 2/9855, 3/9856) the most obvious symptom was a failure of some affected branches to leaf out at all, whilst others had leafed out but had produced unhealthy-looking leaves of a reduced sized compared to healthy branches (see appendix 1, photographs 1 and 2). There were often branches of all three types (healthy, reduced leaves, no leaves) on the same plant.

There were no other external symptoms such as bark loss or discolouration on affected branches, but cutting into them revealed a firm, brown decay of the wood apparently progressing upwards from the base. There was also a dark brown decay of the internal pith, often progressing somewhat further up the branch than the associated wood decay (see appendix 1, photograph 4).

Occasionally it was noticeable that the causal pathogen had been able to colonise higher up the branch, via a wound such as that created by harvesting machinery. Similar internal symptoms of wood and pith decay extended from this point of colonisation (see appendix 1, photograph 8).

When affected plants were dug to take back to the laboratory it was also noticed that there was often a decay affecting parts of the crown (usually the area immediately below the affected branches). In some cases root decay was also evident.

At the site visited in July (4/15816) and in the sample received in August (3/16411) there were similar symptoms present to those described above, but an additional symptom was the wilting and death of foliage on some branches that had initially leafed out in spring (either with reduced leaves or with full-sized leaves) (see appendix 1, photograph 3, taken by Rob Saunders of GSK in July 2009). The dead leaves often remained attached to the branch. All of the wilted branches had typical wood and pith decay at the base.

The severity and distribution of infection varied between sites. At the worst-affected site infected plants were distributed throughout the plantation and up to 80% of plants showed symptoms. At other sites the level of infection was lower and there were sometimes patches (foci) of affected plants.

1.2 Stoolbed sites

There were no obvious symptoms of dieback at sites 5/27999, 6/28580 and 6/28581, and no internal symptoms of decay were apparent at the base of the stems at sites 5/27999 and 6/28581 when these were randomly sampled. At site 6/28580 it was noticed during sampling that a low percentage of stems had decay of the wood or pith at the base.

At sites 5/28000 and 5/28001 it was noticed that there were dead, detached stems lying at the base of some of the plant, and a few dead stems still loosely attached to the crown of the plants. When taking the random stem samples from these sites a low percentage showed internal decay of the pith and/or wood at the base, similar to that seen in affected plants at the plantation sites.

2 Laboratory processing of samples

2.1 Fruiting plantations

See appendix 1, photographs 5, 6 and 7.

Detailed visual examination of the plants from the various sites revealed that the decay affecting the base of the branches was often closely linked to crown decay. Where root decay was present, this usually affected the top few centimetres of the root immediately below the crown and was again closely linked to the areas of crown decay. The fact that lower parts of the root system were unaffected suggested that the causal pathogen had entered the roots from the crown, rather than progressing into the crown from the roots (the latter might be expected if a root pathogen such as *Phytophthora* were responsible).

Microscopic examination of the decayed areas of pith in affected branches revealed that this almost always contained fine fungal mycelium.

There was no immediate evidence of fungal spores or fruiting bodies on most of the plants examined, but occasionally fruiting bodies producing spores of either *Phomopsis* or *Diaporthe* were noted on the branch bases (usually arising from the lenticels - air pores) or, on one occasion, on the areas of crown decay.

2.1.1 Aseptic isolations

A range of fungi was isolated onto agar from the branch, crown and root decay at the various sites. Most of these were secondary colonisers of the decayed material. However, one fungus was isolated consistently from all sites from the decayed wood and pith of affected branches. This fungus produced white colonies with tufts of aerial mycelium on both ¼PDA + P&S and PDA (although growth on the former was sparser) – see appendix 1, photograph 9. This growth habit was suggestive of *Phomopsis/Diaporthe*, although the cultures were reluctant to produce spores and some other fungi (e.g. *Pestalotiopsis*) have a similar growth habit. However, subsequent DNA sequencing results (see results section 3 – Identification of potential pathogens) confirmed that the fungus was *Phomopsis/Diaporthe*.

The same fungus was also isolated from both decayed crown tissue and decayed roots, although with less consistency than from the branches (secondary organisms, which are likely to have colonised the decaying crowns and roots from the soil, were isolated very commonly from these parts of the plants).

A second fungus was isolated quite consistently from the affected branches of plants at site 1/9853 (in addition to the *Phomopsis/Diaporthe* described above). This produced copious quantities of very dark mycelium.

No *Phytophthora* was recovered from the roots or crowns using PARPH agar.

2.1.2 Humid incubation

Incubation of sections of decayed branch material often resulted in the rapid (within 24-48 hours) production of dense tufts of white fungal mycelium from exposed areas of the decayed wood. Picking this mycelium off and plating it onto agar again resulted in growth of *Phomopsis/Diaporthe*.

Prolonged incubation (2-4 weeks) of the branch material led in many cases to the production of black fruiting bodies (pycnidia) of *Phomopsis*, frequently from the lenticels but also on the surface of the decayed wood and pith where this was exposed by splitting open the branch sections. *Phomopsis* produces two spore types called α-spores and β-spores, and there was variation as to which spore type was produced from the fruiting bodies.

After several weeks of further incubation the fruiting bodies of *Phomopsis* were gradually replaced by those of the sexual state of the fungus *Diaporthe*. These fruiting bodies produced ascospores (sexual spores) and whilst also black, developed conspicuously long necks (1-2mm in length) – see appendix 1, photographs 10 & 11. The morphology of the fruiting bodies and spores suggested that the species could be *Diaporthe strumella* (Ellis and Ellis, 1997).

Branch sections from site 1/9853 produced fruiting bodies of a second fungus, in approximately equal quantity to those of the *Phomopsis/Diaporthe.* The spores produced by this second fungus suggested that it could be a *Botryosphaeria* species, and that this could be the fungus producing colonies with dark mycelium that were isolated onto agar from the branches at this site (see above).

Incubation of crowns and roots of plants from the various sites gave more variable results, due to the presence of numerous secondary organisms as described above. However, fruiting bodies of *Phomopsis* and/or *Diaporthe* also sometimes developed on decayed crown or root material.

2.1.3 Baiting techniques

A *Phytophthora* species was recovered from the roots of sample 2/9854 using the rhododendron bait technique. However, no *Phytophthora* was recovered from the crown of these plants, or from the roots or crowns of plants from any of the other sites.

2.1.4 Lateral flow devices (LFD's)

No positive results were obtained from the roots or crowns of plants at any of the sites when these were tested for *Phytophthora* using lateral flow devices.

2.2 Stoolbeds

Results of the initial examinations and subsequent testing of samples from the stoolbed sites are given in tables 3-7.

Table 3 Stoolbed site 5/27999

Type of basal decay present on initial examination	No. of affected stems	Mycelium in pith?	No. of affected stems from which *Phomopsis/Diaporthe* was recovered
Wood decay only	0	N/A	N/A
Pith decay only	0	N/A	N/A
Wood and pith decay	0	N/A	N/A
No decay	20	N/A	1*

*A small patch of fruiting bodies developed about halfway up one stem after approximately three weeks incubation. The fruiting bodies were confined to the bark only – there was no underlying decay. Isolation of these onto agar resulted in colonies of *Phomopsis/Diaporthe* (but see DNA sequencing results below).

Table 4 Stoolbed site 5/27800

Type of basal decay present on initial examination	No. of stems affected	Mycelium seen in pith?	No. of affected stems from which *Phomopsis/Diaporthe* was recovered
Wood decay only	3	N/A	0
Pith decay only	3	No	0
Wood and pith decay	5	Yes – 4/5 No – 1/5	3*
No decay	139	N/A	0

*One stem had fruiting bodies (pycnidia) of *Phomopsis* present on initial examination.

The three stems with wood and pith decay from which *Phomopsis/Diaporthe* was recovered had decay extending 52mm, 160mm and 280mm up the stem from the base, respectively (see appendix 1, photograph 12). Each of these stems had mycelium in the pith. The latter stem was one which was almost dead but still attached to the crown of the plant.

Testing of the dead, detached stems lying at the base of some plants at this site also resulted in consistent recovery of *Phomopsis/Diaporthe*. Many of these stems had numerous fruiting bodies (perithecia) of the *Diaporthe* stage visible on initial examination.

Table 5 Stoolbed site 5/28001

Type of basal decay present on initial examination	No. of stems affected	Mycelium seen in pith?	No. of affected stems from which *Phomopsis/Diaporthe* was recovered
Wood decay only	0	N/A	N/A
Pith decay only*	6	No	0
Wood and pith decay	3	Yes – 2/3 No – 1/3	2
No decay	141	N/A	3**

*See appendix 1, photograph 13.

* *Fruiting bodies (pycnidia) of *Phomopsis* developed on these stems after three to five weeks incubation. They were confined to the bark and developed at various distances from the base of the branch (20mm, 140mm and 260mm, respectively).
The two stems with wood and pith decay from which *Phomopsis/Diaporthe* was recovered had decay extending 23mm, 68mm up the stem from the base, respectively. Both of these stems had mycelium in the pith.

Testing of the dead, detached stems lying at the base of some plants at this site also resulted in consistent recovery of *Phomopsis/Diaporthe*. Some of these stems had fruiting bodies (pycnidia) of *Phomopsis* visible on initial examination.

Table 6 Stoolbed site 6/28580

Type of basal decay present on initial examination	No. of stems affected	Mycelium seen in pith?	No. of affected stems from which *Phomopsis/Diaporthe* was recovered
Wood decay only	0	N/A	N/A
Pith decay only	1	No	0
Wood and pith decay	4	Yes – 4/4	1*
No decay	95	N/A	0

**Botrytis cinerea* (grey mould) was isolated consistently from the other three branches. Resting bodies (sclerotia) of *Botrytis* were present on the bark on initial examination of these stems. Grey mould is a recognised cause of branch dieback (Berrie, undated). The stem from which *Phomopsis/Diaporthe* was recovered appeared to have died back earlier in the summer. This branch also had *Botrytis* present.

Table 7 Stoolbed site 6/28581

Type of basal decay present on initial examination	No. of stems affected	Mycelium seen in pith?	No. of affected stems from which *Phomopsis/Diaporthe* was recovered
Wood decay only	0	N/A	N/A
Pith decay only	0	N/A	N/A
Wood and pith decay	0	N/A	N/A
No decay	95	N/A	1*

* An initial assessment carried out three weeks after the incubation tests were set up revealed no evidence of *Phomopsis/Diaporthe.* However, a second assessment carried out six weeks later revealed that one stem had produced fruiting bodies (perithecia) of *Diaporthe*, approximately 15cm above the stem base. This branch had also developed typical symptoms of wood and pith decay at the base.

3. Identification of potential pathogens

Initial identification results on the fungi recovered from the plantation and stoolbed plants are reported above. These were based on the morphology of fruiting bodies and spores. Apart from a tentative identification of *Diaporthe strumella,* based on the fruiting bodies produced in the humid incubation chambers of some of the material, the precise species of *Phomopsis/Diaporthe* involved was uncertain. It was also unclear whether the same species of *Phomopsis/Diaporthe* was present at all sites or whether different species could be involved.

A number of cultures of the *Phomopsis/Diaporthe* isolated from the plantation and stoolbed sites were sent for DNA sequencing during the course of the project. The dark fungus isolated from site 1/9853 (thought to be a *Botryosphaeria* species) was also sequenced. The results obtained are shown in table 8.

Table 8 DNA sequencing results

Site/Culture reference	Comments	DNA sequencing result
1/9852	Isolated from branches of plantation material	*Phomopsis/Diaporthe* sp. 1
1/9853A	Isolated from branches of plantation material – white colony	*Phomopsis/Diaporthe* sp. 1
1/9853B	Isolated from branches of plantation material – dark colony	*Botryosphaeria obtusa*
2/9854	Isolated from branches of plantation material	*Phomopsis/Diaporthe* sp. 1
2/9855	Isolated from branches of plantation material	*Phomopsis/Diaporthe* sp. 1
3/9856	Isolated from branches of plantation material	*Phomopsis/Diaporthe* sp. 1
4/15816	Isolated from branches of plantation material	*Phomopsis/Diaporthe* sp. 1
5/27999	Isolated from small patch of fruiting bodies that developed on bark of stoolbed stem (healthy on initial examination) during the incubation test	*Phomopsis/Diaporthe* sp. 2
5/28000A	Isolated from wood/pith decay of stem base (present on initial examination) of stoolbed sample	*Phomopsis/Diaporthe* sp. 1
5/28000B	Isolated from dead branch at the base of stoolbed plants	*Phomopsis/Diaporthe* sp. 1
5/28001A	Isolated from wood/pith decay of stem base (present on initial examination) of stoolbed sample	*Phomopsis/Diaporthe* sp. 1
5/28001B	Isolated from dead branch at the base of stoolbed plants	*Phomopsis/Diaporthe* sp. 1
6/28580	Isolated from wood/pith decay of stem base (present on initial examination) of stoolbed sample	*Phomopsis/Diaporthe* sp. 1
6/28581	Isolated from wood/pith decay of stem base (healthy on initial examination) of stoolbed sample. Symptoms developed during the incubation test	*Phomopsis/Diaporthe* sp. 1

Unfortunately, whilst the DNA sequencing confirmed that the cultures were all *Phomopsis/Diaporthe* (with the exception of the *Botryosphaeria obtusa*, which was also clearly different based on culture and spore morphology), there were no close species matches on the DNA database, Genbank. There are currently only a limited number of DNA sequences of *Phomopsis/Diaporthe* species on this database. However, alignment of the sequences for the different isolates showed that, with the exception of isolate 5/27999, all of the other isolates were identical. This means that, with the exception of 5/27999, the isolates from the various plantation and stoolbed sites all belong to the same *Phomopsis/Diaporthe* species.

Agar cultures and branch sections (on which fruiting bodies of the *Diaporthe* state were present) from site 2/9854 were also sent for examination by Dr Paul Cannon of CABI Bioscience (Egham, Surrey), Dr Jutta Gabler of the Julius-Kühn Institute (Germany) and Dr Gerard van Leeuwen of the Netherlands Plant Protection Service. Dr Cannon and Dr van Leeuwun also carried out DNA analyses but were again unable to obtain any close matches

with available DNA sequences of *Phomopsis/Diaporthe* species. Dr Cannon and Dr Gabler examined the morphological features of the fungus and agreed that the species was most likely to be *Diaporthe strumella* (asexual state *Phomopsis ribicola*).

4. Host inoculation tests

4.1 Inoculation of potted plants

Plants were inoculated on 11/6/2010 according to the techniques described in the methods section, using cultures of *Diaporthe strumella* obtained from the following plantation sites:

1/9852
1/9853A
2/9854
2/9855
3/9856

A plant was also inoculated with *Botryosphaeria obtusa* culture 1/9853B as this fungus is associated with dieback problems on a range of woody plants (Sinclair and Lyon, 2005) and a related species (*Botryosphaeria ribis*) has been previously associated with dieback in blackcurrant (Berrie, undated).

No external symptoms of branch dieback or wilting have so far been observed in the test plants between the date of inoculation and the time of writing this report (early April 2010, when the plants have just begun to leaf out).

Branches from one of the wound-inoculation techniques applied to the woody branches were harvested and examined on 25/8/2010, approximately seven weeks after inoculation. On these branches the wound inoculations had been wrapped in damp cotton wool and clingfilm. The branches were assessed for any penetration of decay into the tissues away from the inoculation point. Where decay was present, isolations were made onto ¼PDA + P&S (following surface sterilisation) to see if the fungus used for the inoculation could be recovered. Isolations were made from the decayed tissues at a point well away from the actual inoculation site. Results are shown in table 9.

Table 9. Results of wound inoculation of woody branches (cotton wool / clingfilm), assessed 25/8/2010

Site/culture reference	Decay present?	Spread of decay (mm) from inoculation point		*D. strumella* re-isolated?
		Up branch	Down branch	
Control	No	N/A	N/A	N/A
1/9852	Yes*	25	10	Yes
1/9853A	Yes*	10	15	No
1/9853B (*Botryosphaeria)*	No	N/A	N/A	N/A
2/9854	Yes*	5	20	Yes
2/9855	Yes*	10	10	Yes
3/9856	Yes*	5	5	Yes

*****In all cases where decay was present, this took the form of a dark, narrow zone of decay, approximately 2-3mm wide, confined to the xylem/cambium region and immediately adjacent to the point where the fungal mycelium on the agar block was placed into contact with the plant tissues.

These results indicate that at the time of the assessment the *D. strumella* was beginning to invade the branches from the inoculation point (as evidenced by the fact that it was successfully re-isolated from the decayed areas in all cases except 1/9853A).

A further set of wound-inoculated woody branches (on these the inoculated areas had been wrapped in damp cotton wool and parafilm) were harvested on 25/3/2011, approximately nine and a half months after inoculation. These were assessed as described above, and the results are shown in table 10.

Table 10. Results of wound inoculation of woody branches (cotton wool / parafilm), assessed 25/3/2011.

Site/culture reference	Decay present?	Spread of decay (mm) from inoculation point		*D. strumella* re-isolated?
		Up branch	Down branch	
Control	No	N/A	N/A	N/A
1/9852	Yes**	30	30	Yes
1/9853A	Yes*	30	200	Yes
1/9853B (*Botryosphaeria)*	Yes	3	3	N/A *Botryosphaeria* not re-isolated
2/9854	Yes**	20	40	Yes
2/9855	Yes**	20	45	Yes
3/9856	Yes**	15	10	No

*The decay caused by isolate 1/9853A (see appendix 1, photographs 14 & 15) affected the wood and pith and the symptoms were identical to those seen in the field in plants affected by the dieback problem (including the presence of mycelium in the decaying pith). The decay progressed down beyond the point where the branch was removed at the base, and may have been entering the crown. The plant is being monitored for further development of symptoms.

**Decay caused by the other *D. strumella* isolates was less extensive and mainly confined to the wood, although in some cases the fungus appeared to be entering the pith close to the inoculation point (see appendix 1, photograph 16).

The decay present in these branches is clearly more extensive than that in the branches assessed on 25/8/2010. This is most likely because the fungus has had more time to colonise the branches, although the inoculation technique was also slightly different (parafilm used rather than clingfilm).

The final set of woody branch inoculations (wrapped in damp cotton wool, clingfilm and black plastic) has been left *in situ* on the plants for long-term monitoring (to check, for example, whether the plants develop external symptoms of dieback and/or wilting, in addition to the internal decay described above).

Woody branches inoculated using technique 2 as described in the methods section (pieces of culture placed on unwounded bark) were also harvested and assessed on 25/3/2011. No colonisation or decay had occurred with any of the isolates.

Green, current year's shoots inoculated using technique 3 as described in the methods section (leaf removed, culture placed over wound) were also harvested and assessed on 25/3/2011. By this time the shoots had become woody. No colonisation or decay had occurred with any of the isolates.

The wound inoculation test in which spores were applied (technique 4 as described in the methods section) was set up on 25/8/2010, using spores from isolate 2/9854. Branches were harvested and assessed on 25/3/2011, seven months after inoculation. Neither the *Phomopsis* nor the *Diaporthe* spores had produced any colonisation or decay of the branches.

4.2 Inoculation of detached branch material

The tests on Ben Avon branches were set up on 16/12/10 using *D. strumella* isolate 2/9854 and on 24/12/10 using isolate 5/28000A.

Assessments of the inoculations using isolate 1/9854 were made on 4/2/2011 (after about six weeks). Where decay was present affected tissue (taken well away from the inoculation point) was isolated onto ¼PDA + P&S following surface sterilisation.

One of the branches inoculated using technique 1 (wound inoculation below a 'tongue' of bark) showed decay of the bark and wood, although at the time of the assessment the fungus had not entered the pith. The decay extended 25mm up and 30mm down the branch from the inoculation point. The fungus was successfully re-isolated from this branch. No decay developed on the second inoculated branch, or on the control branch.

Both branches inoculated using technique 2 (wound inoculation by removing a section of bark) showed decay of the bark and wood, although again at the time of the assessment the fungus had not entered the pith (see appendix 1, photograph 18). The decay extended both up and down the branch from the inoculation point (branch 1: 35mm up, 10mm down; branch 2: 35mm up, 15mm down). Isolations were made from branch 1 and the fungus successfully re-isolated from the wood and pith. No decay developed in the control branch.

Both branches inoculated using technique 3 (pith at base removed and replaced with a small piece of agar) showed extensive decay of the wood and pith (see appendix 1, photograph 17), extending up the branch from the inoculation point (to a distance of 210mm in branch 1 and 270mm in branch 2). These symptoms are identical to those seen in the

field in plants affected by the dieback problem (including the presence of mycelium in the decaying pith). The fungus was successfully re-isolated from both the wood and pith of both of these branches. No decay developed in the control branch.

Both branches inoculated using technique 4 (agar block placed on unwounded bark) developed black ‘zone lines’ on the bark (see appendix 1, photograph 19), suggesting that the fungus had been able to colonise the bark. These zone lines were present up to 80mm away from the inoculation point on branch 1 and up to 30mm away on branch 2. Fruiting bodies of *Phomopsis* also developed on the bark of branch 2. There was no decay of the underlying tissues in either branch. The fungus was successfully re-isolated from both branches. No decay developed in the control branch.

The addition in this test of damp paper towel to the seed tray in which the branches were placed also led to extensive growth of secondary fungi over the surface of the branches. This made assessment difficult, and meant that it was not possible to extend the incubation period beyond the first assessment, for example to check whether the colonisation of bark from the unwounded inoculations (technique 4) would spread from here into the internal tissues.

Assessment of the branches inoculated with isolate 5/28000A was not possible due to prolific growth of secondary fungi.

Because of this problem, a further test was set up on 18/2/2011, using Ben Tirran branch sections, in which damp paper towel was not placed in the seed tray prior to incubation. Isolates 2/9854 and 5/28000A were used and inoculated using technique 3 (basal pith inoculation). The test was assessed on 28/3/2011, approximately five weeks after inoculation. All of the branch sections inoculated with the fungus showed extensive decay of the wood and pith, extending upwards from the inoculation point. Again, these symptoms are identical to those seen in the field on plants affected by the dieback problem (including mycelium in the pith). Further details are given in table 11.

Table 11. Results of basal pith inoculations of Ben Tirran detached branch sections, assessed 28/3/2011

Site/culture reference	Inoculated branch number	Progression of decay up branch from base (mm)	*D. strumella* re-isolated?
Control	1	0	N/A
Control	2	0	N/A
Control	3	0	N/A
2/9854	1	300	Yes
2/9854	2	300	Yes
2/9854	3	260	Yes
5/28000A	1	100	Yes
5/28000A	2	80	Yes
5/28000A	3	20	Yes

It can be seen that isolate 2/9854 had colonised the branch tissues more rapidly than isolate 5/28000A (all of the branches were incubated in the same seed tray, and would therefore have experienced identical temperature and humidity).

Due to the lower humidity within the incubation chamber there was much less growth of secondary fungi over the surface of the branch sections. However, it was noticeable that where the bases of the inoculated branches had begun to decay there was extensive production of fruiting bodies and spores of *Botryosphaeria obtusa* over the bark surface. This did not occur on the bases of the control branches and the *Botryosphaeria* was not isolated from the decayed wood or pith in the inoculated branches. It is assumed that the *Botryosphaeria* was acting as an endophyte, i.e. a fungus residing in the bark, which was only triggered to produce spores when the branch began to decay. This might explain why the same species was isolated quite readily from branches from one of the affected plantations (1/9853B), but did not prove to be pathogenic when used in the potted plant inoculation tests.

5 Tests to determine cardinal temperatures of Diaporthe strumella

The tests were carried out using the following isolates of *D. strumella*:

1/9852
1/9853A
2/9854
2/9855
3/9856
4/15816

The growth rates over the course of the test were very similar for all isolates. The charts below show the results for three of the isolates, illustrating this similarity. Figure 1 shows the colony diameters after six days growth, highlighting the optimal growth rate.

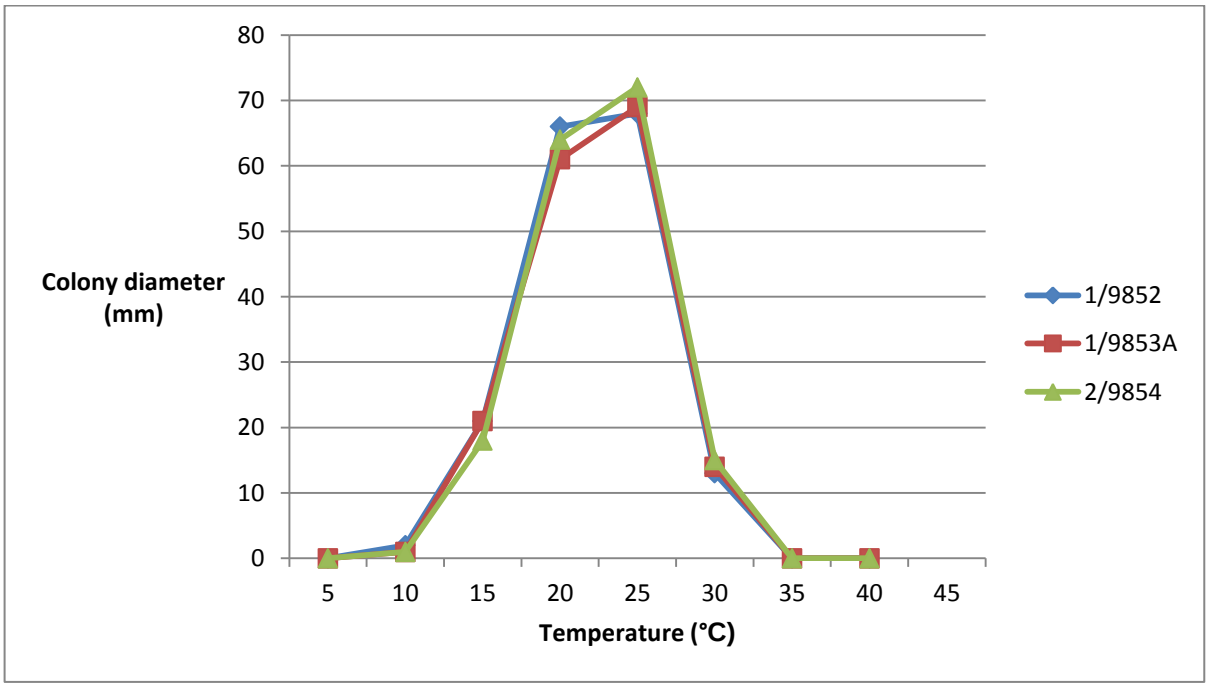

Figure 1. Colony diameter of three isolates *D. strumella* after six days growth.

Figure 2 shows colony diameters after 20 days growth. By this time the growth of the isolates had reached the edge of the agar plate at 15, 20 and 25°C. However, the chart shows that some growth had occurred at 5°C by this time, whereas there was still no growth at 35 or 40°C.

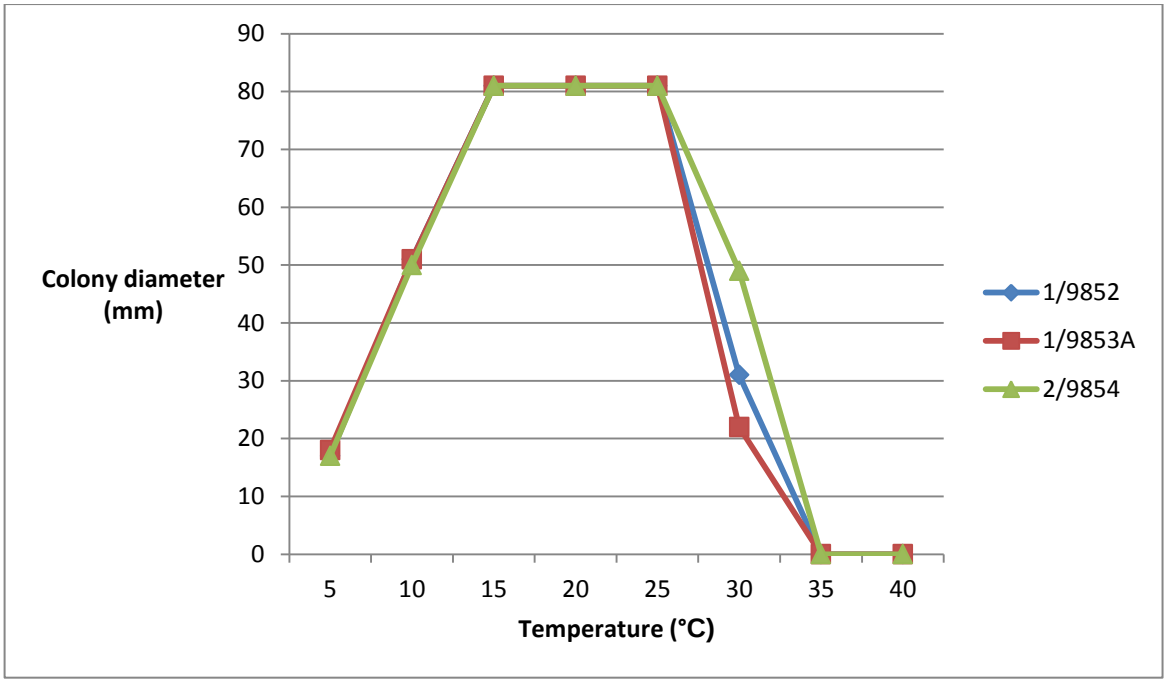

Figure 2. Colony diameter of three isolates of *D. strumella* after 20 days growth.

For all isolates, the minimum temperature for growth was <5°C. Optimal growth temperature was around 25°C, with a sharp decline in growth rate between 25 and 30°C. The maximum temperature for growth was between 30 and 35°C.

6. In vitro tests of fungicide activity against Diaporthe strumella

It was only possible within the budget available for the project to carry out fungicide screening at a fairly basic level, using limited numbers of replicates and fungicide rates. Due to the limited number of replicates used in these tests, it was not possible to use statistical analyses to highlight significant differences between treatments. However, the results provide an indication of potential candidate products to carry forward into field trials.

6.1 Mycelial growth assay

The EC_{50} values for the fungicides tested are shown in table 12.

Table 12. EC_{50} values (parts per million) from mycelial growth assay on agar plates.

Fungicide	Active ingredient(s)	Isolate			
		1/9852	**2/9854**	**3/9856**	**5/28000A**
Bravo 500	chlorothalonil	2.44	0.43	0.24	1.91
Cercobin	thiophanate-methyl	27.2 (11.1)*	37.4 (11.1)*	35.5 (9.8)*	48.4 (16.1)*
Signum	pyraclostrobin + boscalid	1.47	1.47	1.07	0.82
Switch	cyprodonil + fludioxonil	<0.1	<0.1	<0.1	<0.1
Systhane	myclobutanil	0.37	0.33	0.17	0.46

*EC_{50} values are usually calculated by extrapolation from a dose-response curve, obtained by plotting logarithmic conversions of the figures of percentage inhibition of growth at each fungicide concentration. With Cercobin, however, there was little inhibition of growth at the lower fungicide concentrations, but a rapid increase in percentage inhibition between the 10ppm and 100ppm treatments. This resulted in an atypical dose-response curve, giving unreliable EC_{50} values (these are the figures quoted in brackets). A more accurate measurement of the likely EC_{50} could be obtained in this case by plotting the unconverted figures for percentage inhibition, and this is the main figure quoted. However, in order to obtain an accurate EC_{50} for Cercobin, the work would need to be repeated using a detailed series of fungicide concentrations between 10 and 100ppm.

Switch is clearly the most effective fungicide against mycelial growth of the *D. strumella*, whilst Bravo 500, Signum and Systhane are also moderately effective.

6.2 Spore germination assay

The results for this assay must be treated with caution, as there were often quite large differences in absorbance readings between replicates of the same isolate. In some cases this was found to be due to growth of bacteria within the spore solution, leading to higher absorbance readings than would be expected. The readings for isolate 1/9852, and across all isolates for the fungicides Signum and Systhane, were too variable to be included in the results. EC_{50} values for the other fungicides and isolates are given in table 13.

Table 13. EC_{50} values (parts per million) from photometric spore germination assay.

Fungicide	Active ingredient(s)	Isolate		
		2/9854	3/9856	5/28000A
Bravo 500	chlorothalonil	3.35	1.24	0.82
Cercobin	thiophanate-methyl	3.80	6.45	3.31
Switch	pyraclostrobin + boscalid	4.65	1.98	0.92

Results were more variable between isolates, and EC_{50} values were generally higher than for those obtained in the mycelial growth assay.

Discussion

Causes of dieback in blackcurrant

Berrie (undated) lists the following fungi as causes of branch dieback in blackcurrants:

Botrytis cinerea (grey mould)
Nectria cinnabarina (coral spot)
Diaporthe (*Phomopsis*) sp.
Botryosphaeria ribis (Botryosphaeria dieback)
Chondrostereum purpureum (silver leaf)
Eutypa lata (Eutypa dieback or dead arm).
Verticillium dahliae (Verticillium wilt)
Armillaria mellea (honey fungus)

Of these pathogens, the first five cause dieback by attacking the branches directly. *Verticillium dahliae* causes dieback by invading the plant through the roots and colonising the vascular tissues, causing them to become blocked. *Armillaria mellea* attacks and decays the roots.

In this investigation, no evidence was found of either *Verticillium dahliae* or *Armillaria mellea* on plants in the affected plantations. The plants were also tested for Phytophthora root rot (*Phytophthora* species), as this has been recorded previously on *Ribes* species and some

of the symptoms exhibited by the affected plants at the plantations (root, crown and stem base decay) were quite suggestive of this disease. Whilst *Phytophthora* was found in the roots of an affected plant at plantation site 2/9854, this pathogen is not considered to be the primary cause of the current dieback problem. It was not detected at the other sites and the root symptoms found most commonly on the plants affected by the dieback problem (decay apparently progressing into the top of the main roots from the crown) are not typical of those caused by Phytophthora root rot (where decay usually affects the roots first, and subsequently may spread up into the crown).

Of the remaining pathogens in Berrie's list, grey mould (*Botrytis cinerea*) was found associated with dieback at one of the stoolbeds (6/28581), but not at the remaining plantation or stoolbed sites. *Botryosphaeria* was detected on plants from a plantation site (1/9853) and on some of the branch material used in the detached branch inoculation tests. However, the species was *Botryosphaeria obtusa* rather than *B. ribis*. The potted plant inoculation tests showed that the *B. obtusa* obtained from site 1/9853 was of at best very limited pathogenicity, the inoculation resulting in a lesion nine months after inoculation that extended just 3mm from the inoculation point and from which the *Botryosphaeria* could not be re-isolated.

Of the fungi in Berrie's list, only *Phomopsis/Diaporthe* has been recovered consistently in this investigation (both from plants at affected plantation sites and from potential cutting material at stoolbeds). Berrie's record of *Diaporthe* causing dieback in blackcurrant is given as a personal observation, with no supporting literature quoted. She states that *Diaporthe* 'appears to be occasionally responsible for dieback of shoots...' and '...can be locally significant in some plantations'. There is no mention as to which species might be responsible.

Phomopsis/Diaporthe as the cause of the blackcurrant dieback problem and identification of the species involved

Phomopsis/Diaporthe species are found commonly on a very wide range of woody plants affected by dieback problems (Sinclair and Lyon, 2005). Whilst some species may be aggressive causes of disease others are regarded as opportunist pathogens, colonising plants weakened by other factors or suffering from physical damage (although some of these latter species are still capable of causing extensive damage once they have invaded the tissues of the plant). Many species are also found as saprophytes, colonising dead plant tissues, or as endophytes, residing in the tissues (usually bark) of healthy plants.

Sinclair and Lyon state that aggressive species of *Phomopsis/Diaporthe* may cause lesions that expand at any time of the year except when limited by low winter temperatures. Opportunist species are likely to infect only while the host plant's defences are minimal during dormancy or drought, or following freezing damage or other stress factors.

Identification of the species of *Phomopsis/Diaporthe* associated with the blackcurrant dieback problem has, not surprisingly, proved to be problematical, given the paucity of reliable information concerning a genus that is long overdue taxonomic revision. It was not possible to identify the species using DNA sequencing as only a limited number of sequences for *Phomopsis/Diaporthe* species are currently present on the DNA database, Genbank. However, DNA sequencing did provide the valuable information that, with the exception of a single isolate recovered from stoolbed site 5/27999, all of the sequenced isolates obtained from the different plantation and stoolbed sites belonged to the same species.

Identification based on the morphology of cultures, fruiting bodies and spores of the *Phomopsis* and *Diaporthe* states of the fungus is also less than straightforward. The most recent full monograph of the genus is one dating from 1933 (Wehmeyer, 1933). However, the most likely species involved in this case has been identified from morphological traits by both the author of this report and experts in identification of *Phomopsis* (Dr Paul Cannon of CABI and Dr Jutta Gabler of the Julius-Kühn Institute) as *Diaporthe strumella* (asexual state *Phomopsis ribicola*).

It is hoped that work to be started in the near future by researchers in the Netherlands will result in an accurate re-organisation of the genus, so that identification of *Phomopsis/Diaporthe* species using both DNA sequencing and fungal morphology will be more reliable. However, this work is, unfortunately, likely to take several years to complete.

Ellis and Ellis (1997) describe *D. strumella* as being found commonly on attached, dead branches of *Ribes nigrum, R. rubrum* and *R. uva-crispa*. They do not state whether they consider the fungus to be the primary cause of this dieback or a secondary coloniser of the senescent plant tissues.

In inoculation tests (inoculating both potted plants and detached branch sections with material from agar cultures), *D. strumella* isolated from affected plants at both plantation and stoolbed sites has reproduced the symptoms of wood and pith decay exhibited by plants suffering from the dieback problem. The fungus has also been re-isolated from the

symptoms produced on the inoculated plants. The criteria for Koch's Postulates (see materials and methods, section 4) have therefore been satisfied and it can be concluded that *D. strumella* is capable of causing the symptoms associated with the current dieback problem.

In the host inoculation tests significant decay of the plant tissues occurred only where the branches had been wounded prior to applying the fungus, although superficial colonisation of bark occurred in one of the detached branch tests where pieces of agar culture were applied to unwounded branches.

The inoculation of detached branch sections by removing a small piece of pith from the base and replacing it with a piece of agar culture (see materials and methods, section 4.2) seems to be the most reliable method of reproducing the symptoms of the dieback problem. The differing amounts of decay obtained using isolates 3/9854 and 5/25800A, when the inoculated branches were incubated under identical conditions, also suggests that this test may have potential for screening isolates for their degree of pathogenicity.

Potential sources and infection routes of Diaporthe strumella

At most of the affected plantation sites visited the branch dieback has not become apparent to the grower until two, three or even four years after the cuttings were planted (it is possible, of course, that very low levels of infection had gone unnoticed in the preceding years). Once the problem has become apparent the speed of its spread through the plantation has varied, from slow spread between plants in a patchy distribution to almost the entire plantation showing severe dieback within a year.

At present the initial source of *Diaporthe strumella*, and the methods by which it spreads through a plantation, can only be matters for conjecture (see Suggestions for further work, below).

D. strumella has been found at some of the stoolbed sites, in some cases (e.g. isolate 5/28000A) being isolated from internal symptoms of wood and pith decay typical of those also found on plants at affected plantations. Isolate 5/28000A was also capable of reproducing these symptoms in host inoculation tests. These facts suggest that infected cutting material could be an initial source of the problem. However, it should be stressed that not all of the isolates of *Phomopsis/Diaporthe* recovered from the stoolbed material could be DNA sequenced due to budgetary limitations. It is possible that some of these isolates may have been of a different *Phomopsis/Diaporthe* species, as was the case with

the isolate recovered and sequenced from stoolbed site 5/27999. Neither is it known, without host testing of individual isolates, whether all of the isolates of *D. strumella* recovered from stoolbed material would be capable of causing decay (some were found on the bark only).

Spread of the fungus within the plantation could occur via a number of routes. Spores of *Phomopsis* and *Diaporthe* can be spread by water splash (and possibly also insects), whilst those of the *Diaporthe* state may also be airborne. Both states developed readily during humid incubation tests on affected material, and one or both states were also sometimes found during initial examination of affected plant material prior to these tests being carried out.

The precise time of the year, and the length of time over which each of the spore stages is produced in affected plantations, are currently unknown. It is also unknown whether one or both spore types initiates new infections or how this infection occurs. Observations suggest that at least some of the infection could occur via physical damage to branches (see Appendix 1, photograph 8). Sinclair and Lyon (2005) state that some species of *Phomopsis/Diaporthe* are able to infect via leaf scars at leaf fall.

No infection occurred in a test in this investigation where either *Phomopsis* or *Diaporthe* spores or fruiting bodies were applied to wounded branches, although this may have been because the precise conditions for spore germination and infection had not been reproduced in the test.

Berrie (undated) suggests that infection by *Diaporthe* could occur via the flowers (this is known to occur with the grey mould pathogen, *Botrytis cinerea*). A small number of flowers from some of the affected plantations were tested during this project by humid incubation (this is not reported separately in the methods or results section). No *Phomopsis/Diaporthe* was found, but the test method used soon resulted in the flowers becoming overrun by a large number of different saprophytic fungi, and occasionally also by *Botrytis*.

Observation of affected plants suggests that once the fungus is present within the crown it may be capable of repeatedly spreading into the base of the stems/branches. The plants at one of the plantation sites had been cut to ground level after the first outbreak of the problem, but dieback soon re-occurred, apparently spreading into the new branches from residual infection in the crown. This method of spread could also lead to repeated annual infection of the stems produced on stoolbed plants for cutting material.

As *D. strumella* has been isolated from decaying roots there is also the possibility of spread between plants in the row by root-to-root contact.

The results of the cardinal temperature tests on a number of isolates of *D. strumella* show that the fungus is well adapted to growth and survival under the conditions experienced in UK blackcurrant plantations.

Variation in pathogenicity of isolates of Diaporthe strumella, and interaction with site factors

As mentioned previously, the difference in the rate of decay caused when *Diaporthe strumella* isolates 2/9854 and 5/28000A were inoculated into detached branch sections (incubated under identical conditions) suggests that there could be differences in pathogenicity between isolates of the fungus at different sites (or even within a site). This also raises the question of whether isolates exist that are non-pathogenic (which would have implications for the development of any screening test to detect the presence of the fungus in planting material).

The differences in the severity of the branch dieback problem at different plantation sites could partly be a reflection of this variation in pathogenicity, but there are also likely to be site factors involved. Circumstantial evidence from affected plantations suggests that blackcurrant plants that are under stress (e.g. from drought or poor soil conditions) could be more prone to developing dieback as a result of infection by *D. strumella.* It is known with other woody subjects that plants may be predisposed to infection not just by physical damage but by other adverse factors such as drought or transplanting shock (Sinclair and Lyon, 2005).

It is also possible that certain husbandry techniques used on the plants could have a selective effect on the populations of *D. strumella*, for example by selecting for more aggressive isolates of the fungus. An example of such a factor might be the use of certain agrochemicals such as fungicides.

Control of blackcurrant dieback

It is difficult to give detailed recommendations for control, given that the precise way in which *Diaporthe strumella* infects or spreads between plants is currently unknown. However, some general recommendations can be given based on the results of this project and knowledge of the way some other *Phomopsis/Diaporthe* species affect plants:

- Removal of branches affected by dieback, and the clearing up and disposal of all dead branch material at the bases of the plants, should reduce the likelihood of production of fruiting bodies and spores of both the *Phomopsis* and *Diaporthe* states.

- Avoidance, where possible, of physical damage during cultural operations will reduce the number of wound sites through which *D. strumella* may be able to infect branches.

- Aim to provide the best possible growing conditions so that plants do not come under stress. Avoid planting the varieties most prone to attack (Ben Avon and Ben Tirran) on all but the most suitable sites (for example, avoid sites prone to drought, waterlogging or desiccating winds).

The fungicide screening tests in this project have identified fungicides with activity *in vitro* against *D. strumella*, although field evaluation will also be required. Some of these fungicides are already used routinely in affected plantations against other diseases such as leaf spot, grey mould and powdery mildew, without giving any obvious control of the branch dieback problem. Without detailed knowledge of the epidemiology of the disease, however, it is not known whether these routine spray programmes miss a critical application timing that might give control of the dieback problem.

Other possible causes of branch dieback in blackcurrant

The various other fungal diseases that can result in branch dieback were listed at the beginning of this discussion. Laboratory examination may be required to separate dieback caused by one of these pathogens from that due to *Diaporthe strumella.*

The feeding activities of various pests can also result in shoot or branch dieback. These include the larvae of insect pests such as leopard moth (*Zeuzera pyrina*), currant clearwing

moth (*Synanthedon tipuliformis)* and currant shoot borer (*Lampronia capitella*), which burrow within the internal tissues of the shoot, and mammalian pests such as voles, rabbits and squirrels, which may strip the bark from the branches. The activities of these various pests should be fairly obvious, although the damage that they cause could be another way in which *Diaporthe strumella* is able to colonise branches.

Dieback may also result directly from extensive physical damage to shoots or branches as a result of cultural operations.

A symptom noted in some of the stems sampled from the stoolbeds, which could be confused with those caused by *D. strumella,* was a browning of the pith at the base of the stem (see appendix 1, photograph 13). The cause of this symptom is unknown, although frost damage is one possibility. However, there was no associated wood decay, and it was noticeable that no fungal mycelium was present in the discoloured pith, whereas this is always found in branches affected by *D. strumella*. The presence of mycelium in the pith is not diagnostic for *D. strumella*, however, as it was also seen in branches affected by *Botrytis cinerea.*

Suggestions for further work

Given the limited amount of information currently available on sources, spread and control of blackcurrant dieback, the following would all be valid areas for further investigation:

- Assessment of the timing and duration of spore production and release by the *Phomopsis* and *Diaporthe* states of *D. strumella* in affected plantations.
- Assessment of flowers for colonisation by *D. strumella* (using either ELISA testing (see below) or isolations onto an appropriate agar medium).
- Testing of high-health status cutting material for the presence of *D. strumella*.
- Testing of the crowns of stoolbed plants for colonisation by *D. strumella* (it was not possible to check the crowns of plants from which stems with internal decay were obtained due to severe and penetrating frost at the time of sampling).
- Further more detailed *in vitro* screening of fungicides.
- Field trials of candidate fungicides.
- *In vitro* testing and field trials of biological control organisms. *In vitro* testing is to start shortly, using commercially available biological controls and also a potential biological control organism obtained from plantation material during the course of this project.

- Treatment of cutting material using hot water.
- Selection of cutting material to avoid the most likely points of infection i.e. determine the relative likelihood of infection arising from cuttings selected from the base, middle and top of the stoolbed stems.
- Development of a screening system for cutting material. During this project contact was made with workers at the Julius-Kühn Institute in Germany, who have developed a *Phomopsis/Diaporthe*-specific antiserum for use in ELISA tests. They have carried out a preliminary test of the antiserum on *D. strumella*-affected blackcurrant branch material from one of the plantation sites and have obtained good results. It is possible that the antiserum could be used to screen cutting material for *D. strumella,* although one limitation is that it is genus-specific only i.e. it will also react to other species of *Phomopsis/Diaporthe.* A combination of ELISA testing and visual assessment after incubation may therefore be required.
- Testing of variability between the pathogenicity of isolates using a standard inoculation technique (e.g. the basal pith technique on detached branch sections).
- Detailed genetic work (e.g. microsatellite analysis) to try and determine relationships between isolates of *D. strumella* obtained from the various plantation and stoolbed sites.

Conclusions

- Symptoms of the recent branch dieback problem affecting blackcurrants, particularly the varieties Ben Avon and Ben Tirran, are consistent between plantations.
- External symptoms include wilting and death of individual branches during the summer and in spring a failure of some branches to leaf out, or the production of small, unhealthy-looking leaves.
- Internal symptoms in affected stems or branches include wood and pith decay, usually spreading upwards from the base. Decay of parts of the crown and of the top of the roots is also seen in many cases.
- A *Phomopsis/Diaporthe* species was isolated consistently from affected plants.
- DNA sequencing revealed that the *Phomopsis/Diaporthe* species found at all of the plantation sites, and at all but one of the stoolbed sites, was identical.
- The species has been identified from morphological features as *Diaporthe strumella.*
- Host inoculation tests have shown that *D. strumella* is capable of causing the internal symptoms shown by the branches of plants in affected plantations.

- The inoculation tests have shown that there may be variation in pathogenicity between isolates of *D. strumella*.
- *D. strumella* has also been found at stoolbed sites producing hardwood cutting material. At some sites the fungus was isolated from stems found with internal symptoms identical to those seen at the affected plantations.
- Determination of cardinal temperatures has shown that *D. strumella* is well adapted to UK conditions.
- Screening of fungicides has identified a number of potential candidates for field trials, with Signum (cyprodonil + fludioxonil) appearing particularly effective.

Glossary

α-spores (alpha-spores) and **β-spores (beta-spores)**: types of asexual spore produced by *Phomopsis*. They are formed in a pycnidium and distinguished by their different shapes. Different species or isolates of *Phomopsis* may produce one or both of the spore types. Only α-spores can germinate and infect plants. The precise function of β-spores is unknown.

Ascospores: sexual spores produced by *Diaporthe*, formed in a perithecium.

Cardinal temperatures: the minimum, maximum and optimum temperatures for growth of an organism.

Diaporthe: the sexual state of *Phomopsis.*

EC_{50}: the fungicide concentration at which growth of a fungus is inhibited by 50% compared to its growth in the absence of the fungicide.

An **Isolate**: a pure culture of a micro-organism.

Isolation: the process by which a micro-organism (in this project a fungus) is separated from its substrate (in this project blackcurrant plant material) and established in pure culture.

Perithecium (plural: perithecia): a fruiting body produced by *Diaporthe* in which ascospores are formed. The black perithecia of *Diaporthe strumella* at first appear very similar to the

pycnidia produced by the *Phomopsis* state but go on to develop long, protruding necks (see appendix 1, photographs 10 & 11).

Phomopsis: the asexual state of *Diaporthe.*

Plantation: blackcurrant plants grown for fruit production.

Pycnidium (plural: pycnidia): a fruiting body produced by *Phomopsis*, in which asexual spores are formed.

Stoolbed: blackcurrant plants grown for the production of hardwood cuttings.

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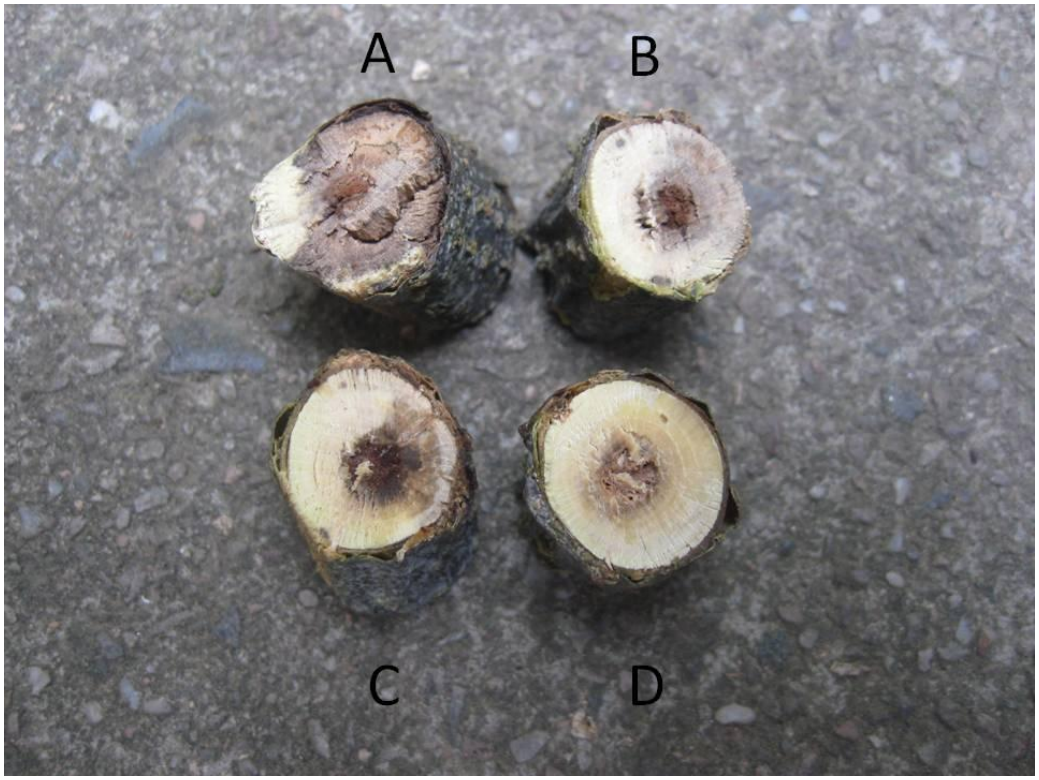
Appendix 1. Photographs

1. Plant of Ben Avon at plantation site 1/9852, showing a branch producing leaves of reduced size (photo taken 13/5/2010).

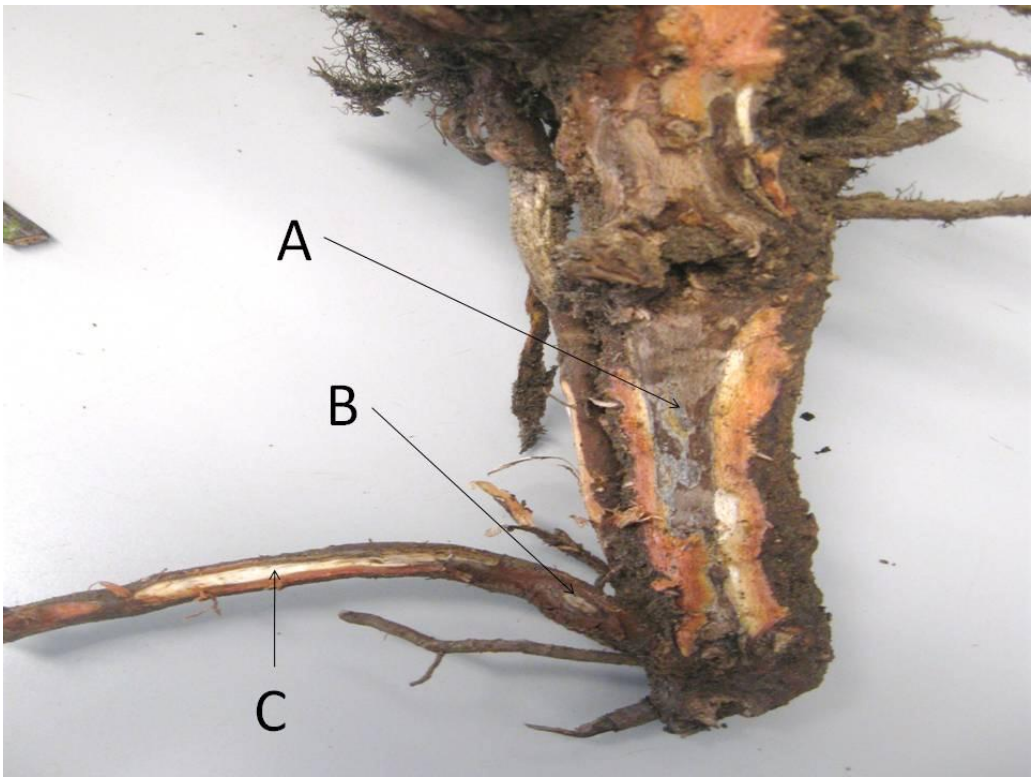
2. Plant of Ben Avon at plantation site 2/9854, showing severe dieback (photo taken 13/5/2010).

3. Plant of Ben Avon at site 2/9854 showing wilting of branches that had initially produced healthy leaves (photo taken July 2009)



4. Ben Avon from plantation site 3/9856, branch sections showing the progression (A to D) of wood and pith decay upwards from base of the branch. A = base of branch D = upper extent of decay (45 cm from base).

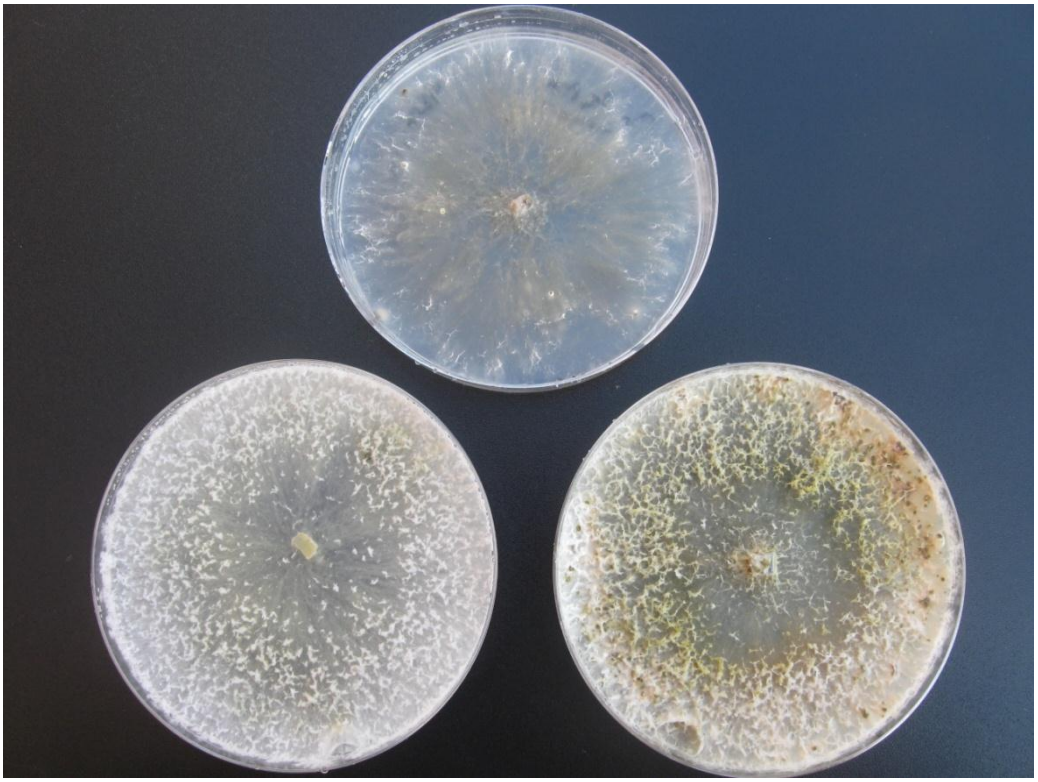
5. Ben Avon, site 3/9856, showing wood decay progressing into stem from side branch



6. Ben Avon, site 3/9856, showing crown and root decay. A = crown decay, B = decay of top of root immediately adjacent to crown decay, C = rest of root unaffected

7. Ben Tirran, site 4/15816, section across the crown, showing decay.

8. Ben Tirran, site 4/15816, branch showing colonisation of wood and pith via a bark wound.

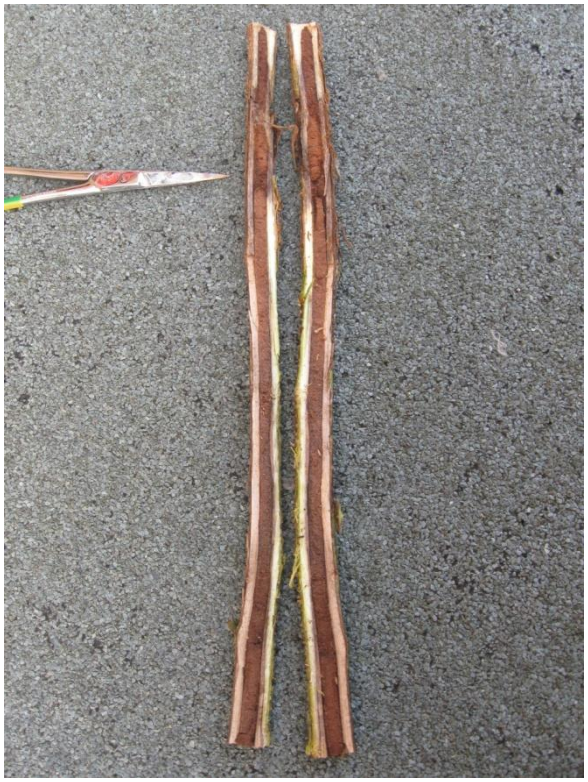


9. Cultures of *Phomopsis/Diaporthe* isolated from blackcurrant samples. Top: 5-week old culture on ¼ PDA + P&S. Bottom left: 2-week old culture on PDA. Bottom right: 5-week old culture on PDA. Parts of older cultures often colour first yellow and then black in places.

10 & 11. Long-necked fruiting bodies (perithecia) of *Diaporthe*, protruding through the lenticels of a branch.

12. Stem sample of Ben Avon from stoolbed site 5/28000, showing wood and pith decay at the base, present at initial assessment - *Phomopsis/Diaporthe* isolated

13. Stem sample of Ben Tirran from stoolbed site 5/28001, showing pith decay only, present at initial assessment – no *Phomopsis/Diaporthe* isolated



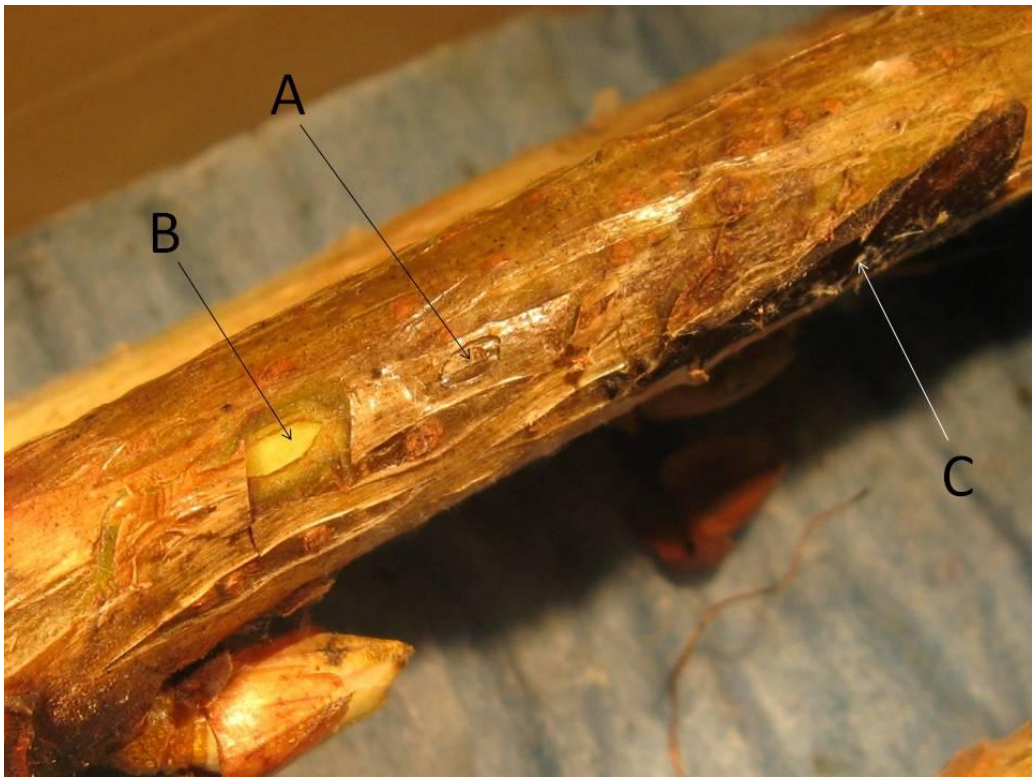
14. Potted plant inoculation, assessment 25/3/2011. Branch inoculated with isolate 1/9853A, split longitudinally to show extensive wood and pith decay (point of scissors at inoculation point).

15. Base where the branch shown in photograph 14 was removed, showing progression of decay below the point of removal.

16. Potted plant inoculation, assessment 25/3/2011. Branch inoculated with isolate 2/9854 (point of scissors at inoculation point), showing decay affecting wood and beginning to enter pith.

17. Detached branch inoculation, assessed 4/2/2011. Branches inoculated with isolate 2/9854, using basal pith technique. Branch sections split longitudinally. Control on left.

18. Detached branch inoculation, assessed 4/2/2011. Branches inoculated with isolate 2/9854, using bark section removal technique. Branch sections showing decay of bark and wood.



19. Detached branch inoculation, assessed 4/2/2011. Branches inoculated with isolate 2/9854, agar placed on unwounded bark. A = black ‘zone lines’. B = bark removed to show lack of penetration. C = remains of agar block.

Appendix 2: Recipes for agars, Petri's solution and Glucose Peptone Medium (GPH)

Potato Dextrose Agar (PDA)

Potato dextrose agar	39g
Distilled water	1.0l

Autoclave at 121°C for 15 minutes.

Quarter-strength Potato Dextrose Agar plus Penicillin and Streptomycin (¼PDA + P&S)

Potato dextrose agar	9.75g
Agar technical no3	5.25g
Distilled water	1.0l

Autoclave at 121°C for 15 minutes.

Antibiotic stock solutions:

0.6% Penicillin G sulphate

Dissolve 0.6g of penicillin sulphate (Sigma P 3032) in 100 mls distilled water. Filter, sterilise and store in a sterile container in a refrigerator. Add 10ml stock solution to each litre of cooled media prior to pouring.

2.0% streptomycin sulphate

Dissolve 2.0g streptomycin sulphate in 100mls distilled water. Filter, sterilise and store in a sterile container in a refrigerator. Add 10ml stock solution to each litre of cooled media prior to pouring.

PARPH Agar

Cornmeal agar	17.0g
Distilled water	1.0l

Autoclave, then cool to 50^{o}C.

Pimaricin	5mg
Ampicillin (Na salt)	250mg
Rifampicin (dissolve in 1ml 95% ethanol)	10mg
PCNB	100mg
Hymexazol	75.0mg

Dissolve the above in 10ml sterile distilled water, then add to cooled autoclaved CMA. Store at 4^{o}C in the dark, use in 1-3 days.

Petri's Mineral Solution

Calcium nitrate ($Ca(NO_3)_2$)	0.4g
Potassium dihydrogen phosphate (KH_2PO_4)	0.15g
Magnesium sulphate ($MgSO_4.7H_20$)	0.15g
Potassium chloride (KCl)	0.06g
Distilled water	1.0l

Glucose Peptone Medium (GPM)

Dextrose	14g
Bactopeptone	7.1g
Yeast extract	1.4g
Distilled water	1.0l

Autoclave at 121°C for 15 minutes.

