Project title:	Phomopsis dieback of blackcurrants: methodology development and control
Project number:	SF 142
Project leader:	Dr Philip Jennings, The Food and Environment Research Agency
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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

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

- Detection of stem dieback using a monoclonal antibody-based test was unreliable. Development of a DNA based detection method is recommended.
- Results indicate spread of stem dieback can be reduced by lowering levels of residual inoculum by removal of previous crop debris
- Reduction in damage to stems reduces risks of infection
- Risks of infection and rate of lesion development increases with temperature reaching an optimum at 20ºC.
- Several of the fungicides known to be effective against stem dieback did not affect growth and sporulation of the potential biocontrol agent *B. leucophthalma*
- Serenade was highly effective against growth and sporulation of Phomopsis ribicola

Background

In recent years dieback caused by *Phomopsis ribicola*, particularly of the varieties Ben Avon and Ben Tirran, has been a significant problem in commercial blackcurrant plantations. The severity of the problem and its speed of spread through a plantation showed significant variation between sites.

Typical symptoms in the spring/early summer include the production of leaves of reduced size or a failure of branches to leaf out. One or both of these symptoms can often be present, along with healthy-looking branches on the same bush. As the summer progresses wilting of leaves may develop and these then generally turn yellow and then brown. Cutting open of symptomatic tissue reveals a firm, brown decay of the wood, with an associated brown decay of the pith at the base of the branch. Tiny, black fungal fruiting bodies may be found on branches that have been dead for some time .In extreme cases, all of the branches on an affected plant may fail to produce leaves.

HDC project SF 12-223 showed that *Diaporthe strumella*, (asexual state *Phomopsis ribicola*), was the only pathogen isolated consistently from blackcurrant plants showing symptoms of dieback that was capable of reproducing dieback symptoms when inoculated into healthy plants. As part of project SF 12-223, visits were also made to stoolbed sites which supplied cutting material for use on fruiting plantations. These visits revealed low levels of *D. strumella* at the majority of stoolbed 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.

At present the sources of the fungus and the route by which it spreads through a plantation are poorly understood. This one year project aimed to develop routine methodologies for the detection of *P. ribicola* in substrates such as plant material, soil and water, develop a better understanding of the infection process and explore potential control strategies in more detail. In the longer term the development of these methodologies would aid the establishment of inoculum sources, routes of infection/spread, and methods of management/control of the disease.

Summary

Evaluation of an antibody-based test for detection of P. ribicola

Initial tests on the Phomopsis genus specific antiserum, Phomopsis-IgG 59/II, showed that extraction from woody stem material was possible, particularly when coating buffer was used as the buffer in the extraction process. The optimal dilution rate for the antiserum was 1:1000. However, use of the optimised test for detection of *P. ribicola* from artificially infected stem material showed results from the test to be inconsistent. It was concluded that the production of PCR primers to detect *P. ribicola* was likely to provide more consistent results.

Development of baiting techniques for improved detection

Three bait types, leaf, green stem and woody stem, were tested for the detection of *P. ribicola* in soil and water. Initial tests carried out in sterile soil and water, primed with differing levels of *P. ribicola* spores, showed that all three bait types detected *P. ribicola* although it was more readily detected in woody stem material. Use of this bait had a lower detection limit of 100 spores/ 75 g soil and 10 spores/ 200 ml water.

When the test was repeated using non-sterile soil or rainwater, the presence of faster growing fungal species, such as fusaria, over-grew *P. ribicola;* as a result the presence or absence of *P. ribicola* in the bait could not be confirmed. Further work would be needed to develop a selective medium for isolation from baits used in field (non-sterile) conditions.

Understanding the infection process in blackcurrant woody stem sections

Stem sections of the blackcurrant variety Ben Avon were tested to determine methods for producing stem infection. Stems were used either wounded or unwounded and inoculated with either a spore suspension or mycelial plug taken from an agar plate of *P. ribicola*. Both methods of inoculation produced symptoms typical of those associated with Phomopsis stem dieback on wounded material. No symptoms were produced on any unwounded material. Lesions produced following inoculation with a spore suspension were larger than those produced following inoculation with a mycelial plug. Observation of the spores after application revealed that spore suspension had been absorbed into the stems and potentially distributed internally within a few hours of inoculation. This is in contrast to the mycelium inoculation would need time to grow into the wound before infection could occur. Continued incubation of the infected stems led to the production of the long-necked fruiting bodies associated with *Diaporthe strumella,* the sexual state of *P. ribicola.* This may indicate that crop debris on the soil surface of blackcurrant plantations could become colonised by *P. ribicola* present in the soil and provide a source of inoculum for subsequent infection of a crop.

The effect of temperature on infection was established using mycelial plug inoculum of *P. ribicola* and wounded woody stem sections of Ben Avon. Seven temperatures (0, 5, 10, 15, 20, 25 and 30°C) and seven exposure times were tested (2 hours, 1, 2, 3, 4, 5 and 6 days). No infection occurred following a 2 hour exposure to *P. ribicola* at any of the temperatures tested. Equally there was no consistent infection at 0 and 5ºC at any of the exposures times tested. Infection rates were also inconsistent for all temperatures where the exposure to inoculum was less than 4 days. At temperatures of 20ºC or higher infections occurred after a 1 day exposure to inoculum; however the level of infection was sporadic until an exposure time of 5 days.

The effect of temperature on symptom extension was tested at temperatures between 0 and 30ºC. Minimal lesion extension was recorded for temperatures 10ºC and below over the 10 day incubation period. For temperatures of 15ºC to 30ºC there was no significant difference in lesion size up to 5 days incubation, however by 7 days incubation the lesion size was significantly smaller at 15 and 30ºC compared to those at 20 and 25ºC. The optimum incubation temperature for lesion extension in woody stem tissue was 20ºC.

These data suggest that where infections occur in blackcurrant stem tissue then lesion expansion will be relatively rapid at temperatures typical of late spring, summer and early autumn in the UK. The expansion is likely to continue, although at a much reduced rate at temperatures more representative of early spring and late autumn. These laboratory tests were carried out on woody stem tissue and will be typical of the host tissue on individual

blackcurrant stems. However, it is likely that where infection occurs at the crown, lesion expansion will be slower due to the denser nature of the tissue.

Effect of fungicide treatments for control of dieback on beneficials (Bloxamia leucophthalma)

B. leucophthalma is a fungus which has been isolated from blackcurrant plantations and has been shown to have *in vitro* biological activity against *P. ribicola*. As a result it would be beneficial to try and ensure that where possible fungicide programmes used to control *P. ribicola* did not have an adverse effect on *B. leucophthalma.*

Tests were carried out to determine whether Signum (boscalid (267 g/kg) + pyraclostrobin (670 g/kg)), Scala (pyrimethanil (400 g/L)), Stroby (kresoxim-methyl (500 g/kg)), Switch (cyprodinil (375 g/kg) + fludioxonil (250 g/kg)), Systhane 20 EW (myclobutanil (200 g/L) or Teldor (fenhexamid (500 g/L)) had any detrimental effect on growth or spore germination of *B. leucophthalma*. EC_{50} values for the fungicides tested showed that Stroby, Teldor and Systhane 20 EW had the least effect on *B. leucophthalma*. Results indicate that there are several fungicides which could be used safely to control of botrytis and leaf spot whilst maintaining the beneficial effects of *B. leucophthalma*.

Efficacy of biocontrol agents (Serenade) against P. ribicola

In HDC project SF 12-223 it was shown that Serenade (a suspension concentrate containing 13.96 g/L *Bacillus subtilis* strain QST 713 at a minimum of $1x10^{12}$ cfu/L) inhibited the growth of *P. ribicola* isolates at the lowest concentration tested (10 ml/L). The work undertaken in SF 12-223 was continued in this project to establish the EC_{50} and minimum inhibitory concentration of Serenade towards *P. ribicola*. The results show that Serenade was highly effective at controlling mycelial growth of *P. ribicola* with EC_{50} values ranging from 0.92×10^{-9} to 8×10^{-9} ml Serenade per litre of agar. The lowest concentration of Serenade that resulted in no growth of the *P. ribicola* was 0.001 ml/L. These data suggest efficacy such that this biocontrol agent could form a key element in an integrated control strategy.

Financial Benefits

The severity of *Phomopsis* dieback varies between blackcurrant plantations, however on one plantation 80% of plants showed symptoms during the course of one year. The cost of losses has been estimated at £5,000 per hectare per year for this plantation alone. Severe attacks have also led to the premature grubbing of plantations.

Establishing an understanding of the pathogen responsible for blackcurrant dieback will ensure effective management strategies can be developed which will minimize future losses from the disease.

Action Points

The aim of this project was to develop methodologies which would allow for potential further work on disease monitoring and epidemiological studies to be carried out. However a number of effective management actions have already been identified from the project;

- Limit wounding to stem material as tissue damage was required for infection to occur.
- Remove crop debris from stoolbeds and the wider blackcurrant plantation as this can provide material on which inoculum can develop to reinfect plants in the following season.

SCIENCE SECTION

Introduction

In recent years dieback, particularly of the varieties Ben Avon and Ben Tirran, has been a significant problem in commercial blackcurrant plantations. The severity of the problem and its speed of spread through a plantation have showed significant variation between sites.

Typical symptoms in the spring/early summer include the production of leaves of reduced size or a failure of branches to leaf out. One or both of these symptoms can often be present, along with healthy-looking branches on the same bush. As the summer progresses wilting of leaves may develop and these then generally turn yellow and then brown. Cutting open of symptomatic tissue reveals a firm, brown decay of the wood, with an associated brown decay of the pith at the base of the branch. Tiny, black fungal fruiting bodies may be found on branches that have been dead for some time. In extreme cases, all of the branches on an affected plant may fail to produce leaves.

HDC project SF 12-223 showed that *Diaporthe strumella* (asexual state *Phomopsis ribicola*) was the only pathogen isolated consistently from blackcurrant plants showing symptoms of dieback that was capable of reproducing dieback symptoms when inoculated into healthy plants. As part of project SF 12-223, visits were also made to stoolbed sites which supplied cutting material for use on fruiting plantations. These visits revealed low levels of *D. strumella* at the majority of stoolbed 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.

At present the sources of the fungus and the route by which it spreads through a plantation are poorly understood.

This one year project aimed to develop routine methodologies for the detection of *P. ribicola* in substrates such as plant material, soil and water and to develop a better understanding of the infection process and explore potential control strategies in more detail. In the longer term the development of these methodologies would aid the establishment of inoculum sources, routes of infection/spread and methods of management/control of the disease.

Materials and methods

1. Detection of, and infection criteria for, *Phomopsis ribicola*
    1.1. Optimisation and validation of a *Phomopsis* genus specific antibody for use in ELISA for the detection of *P. ribicola* from blackcurrant plant material.

Phomopsis-IgG 59/II, a polyclonal genus specific antiserum to *Phomopsis*, was obtained from the Julius Kuhn Institute (Germany) and validated for the detection of *P. ribicola* from infected woody stem tissue. To validate the antibody three different extraction buffers were tested:

(i) 50% Acetic acid;
(ii) Phosphate buffered saline (PBS);
(iii) Coating buffer (pH 9.6).

As there was very little information available for the antibody, including which species it had been raised in, a range of antibody dilutions and both mouse and rabbit anti-species antibodies were included in the validation.

Antigen extraction and microtitre plate coating

Woody stem material, which had previously been artificially infected with *P. ribicola* or used to bait *P. ribicola* from water or soil, was cut into 2 cm sections and placed into a Stomacher sampling bag with 2 mL of an extraction buffer. The stem material was broken down using a rubber mallet, 100µl of the buffer pipetted into wells of a 96 well microtitre plate and incubated at 4°C overnight to coat the antigen onto the plate.

ELISA test

After coating with antigen, each microtitre plate was washed three times with phosphate buffered saline containing added Tween (PBST) ensuring all excess PBST was removed after the final rinse. Blocking buffer (200µl), comprised of PBST + 1% bovine albumin, was added to the each well of the coated plate and incubated at 33°C for 1 hour; this process ensured that any sites not coated with antigen were blocked. After incubation each plate was rinsed using PBST buffer and 200µl of the Phomopsis-IgG 59/II antibody at a range of dilutions (1:100, 1:1000, 1:2000 and 1:4000) in PBS were added to the plate and incubated at 33°C for 1 hour before again being rinsed using PBST. 100µl of anti-species antibody (either mouse or rabbit), diluted 1:4000 in PBST + 0.2% bovine albumin, was added to the

test wells and incubated at 33°C for 1 hour before being rinsed using PBST buffer. Finally, 100µl of p-Nitrophenyl Phosphate Disodium Salt (PNPP), diluted to 1mg/ml in PBS buffer, was added to the plate and incubated for 1 hour at room temperature before absorbance of each well was measured at 405 nm using a spectrophotometer.

1.2. Establish reliable methods for the detection of *P. ribicola* from water and soil samples.

To carry out epidemiological study on a pathogen it is essential to be able to detect the pathogen from soil and or water samples. One way of detecting the pathogen in these substrates is through baiting. Three bait types, blackcurrant leaves, green stem and woody stem, were tested for their effectiveness at baiting *P. ribicola* spores in soil and water. Prior to testing all bait material was autoclaved at 110°C for 10 minutes.

Baiting from water

Tests for each bait type were set up in 1 L plastic microwavable tubs. For each bait type three replicate tubs were set up containing 200 ml water with either 0.05, 0.5, 5, 50, 500 or 5000 pycnidia ml^{-1} (10, 100, 1000, 10,000, 100,000 and 1,000,000 spores total). Pycnidia were harvested directly from the surface of 14 day old colonies of *P. ribicola* grown on PDA. Tubs containing 200 ml water only were used as controls. Two separate tests were set up, one using sterilised water (autoclaved at 121ºC for 15 minutes) and the other using unsterilised water. Four replicate pieces of bait material were used to test each bait type. All tubs were left at 20ºC and after seven days the bait material removed and plated onto PDA. Plates were incubated for a further 7 days and assessed visually for growth of *P. ribicola*.

Baiting from soil

For each bait type three replicate tubs (as described above) were set up containing 75 g soil, with total spore numbers the same as those used in the water test. Pycnidia were harvested as previously described and added to the soil in 5 ml water. Tubs containing 75 g soil with no spores added were used as controls. Two separate tests were set up, one using sterilised soil (autoclaved at 121ºC for 15 minutes on three consecutive days) and the other using unsterilised soil. Four replicate pieces of bait material were used to test each bait type. All tubs were left at 20ºC and after 7 days the bait material removed and plated onto PDA. Plates were incubated for a further 7 days and assessed visually for growth of *P. ribicola*.

2. Investigation of the infection process

2.1. Development of methodologies for infecting blackcurrant stems with *P. ribicola.*

The ability of *P. ribicola* to infect wounded and unwounded woody stem material through inoculation using mycelial plugs or a droplet of spore suspension was investigated.
Woody stem material (approx. 1cm diameter) of the blackcurrant variety Ben Avon was cut into 5 cm sections and surface sterilised by placing in 70% ethanol for 30 seconds, then a 1% sodium hypochlorite solution for 1 minute and finally 70% ethanol for 30 seconds. Stems were air dried for 10 minutes and the cut ends sealed with Vaseline to prevent contamination. The infection tests were carried out on both wounded and unwounded stems. Stems were wounded taking by either 1) piercing with a drawing pin (ensuring the pin tip entered the pith) or 2) a small incision made into the bark using a scalpel.

Both wounded and unwounded stems were inoculated using:

i) 5 mm agar plugs taken from the leading edge of a 7 day old *P. ribicola* isolate and placed over the wound site. Control stems were inoculated using a 5 mm plug of potato dextrose agar (PDA).
ii) 20 µl of a spore suspension containing 10^6 pycnidia/ml placed directly over the wound site. The spore suspension was produced by washing spores directly from the surface of a 14 day old PDA plate of *P. ribicola*. Control stems were inoculated using a droplet of sterile distilled water (SDW). Unwounded test material was inoculated as described previously with the inoculum placed directly onto the intact bark surface.

All stem material was placed into a damp chamber and incubated at 20°C for 14 days. Stems were then removed from the chamber and split lengthways to assess for disease symptoms.

2.2. Laboratory experiments to determine the optimum temperature for infection, and subsequent symptom development, of blackcurrants by *P. ribicola.*

The optimum temperature and time taken for *P. ribicola* to infection both wounded and unwounded blackcurrant stems was tested at 0, 5, 10, 15, 20, 25 and 30°C. Woody stem material (approx. 1cm diameter) of the blackcurrant variety Ben Avon was cut into 5 cm sections and inoculated using a 5 mm agar plug taken from the leading edge of a 7 day old colony of *P. ribicola*. Where tests were carried out on wounded stems the stems were pierced using a drawing pin, ensuring that the pin tip entered the pith of the stem, and the

agar plug placed directly over the wound site. Inoculated stems were placed into humidity chambers and incubated at the appropriate test temperatures. Four stems were removed from each test temperature after 2 hours, 1, 2, 3, 4, 5 and 6 days, the agar plug removed and the stem surface sterilised by wiping with 70% industrial methylated spirit to ensure that no viable mycelia remained on the stem surface. Stems were then placed into a second humidity chamber and incubated at 20°C for 7 days before splitting them lengthways to assess for disease symptoms. Any lesions found were plated onto PDA to confirm the presence of *P. ribicola*.

The rate of lesion development of *P. ribicola* on blackcurrant woody stem material was tested at temperatures of 0, 5, 10, 15, 20, 25 and 30°C. Stem material was cut into 7.5 cm lengths, wounded using a pin and inoculated by placing 20µl of spore suspension, at a concentration of $1x10^6$ spores/ml, directly over the wound site. Control stems were inoculated using 20 µl of SDW. Stems were placed into a humidity chamber and incubated at 20°C to allow the spores to germinate and begin infection. After 24 hours the stems were removed and split lengthways through the wound site. Ten randomly selected half stems were then placed into humidity chambers and incubated at each of the test temperatures with lesion length measured after 3 ,4, 5, 7, 10 and 12 days.

3. Control
    3.1. Determine the effect of selected fungicides on the potential biocontrol agent *Bloxamia leucophthalma.*

Fungicide sensitivity of *B. leucophthalma* was determined using both an agar plate and a photometric assay to measure the effects of six products commonly used on blackcurrants (Table 1).

Table 1. Fungicides used in efficacy tests against *Bloxamia leucophthalma*

Product	**Active ingredients**
Signum	boscalid (267 g/kg) + pyraclostrobin (670 g/kg)
Scala	pyrimethanil (400 g/L)
Stroby	kresoxim-methyl (500 g/kg)
Switch	cyprodinil (375 g/kg) + fludioxonil (250 g/kg)
Systhane 20 EW	myclobutanil (200 g/L)
Teldor	fenhexamid (500 g/L)

Amended agar assay

This assay was used to determine the effect of fungicides on mycelial growth. A primary screen was carried out on all products using potato dextrose agar (PDA) amended to give total active ingredient concentrations of 10 ppm. Three replicate plates of each concentration were inoculated with a 5 mm agar plug taken from the leading edge of a 7 day old culture of *Bloxamia leucophthalma* and incubated at 24°C. Non-amended PDA plates were used as controls. Colony growth was measured 14 days after inoculation and, based on the inhibition of growth at 10 ppm, a fungicide concentration series selected for each product in order to calculate the EC_{50} (Table 2). Three replicate plates were set up for each isolate and fungicide combination.

Table 2. Total active ingredient (a.i.) concentrations used in the amended agar growth test

Product	**Active ingredient**	**Total a.i. (ppm)**
Signum	boscalid (267 g/kg) + pyraclostrobin (670 g/kg)	50, 25, 10, 5, 1
Scala	pyrimethanil (400 g/L)	10, 5, 1, 0.5, 0.1
Stroby	kresoxim-methyl (500 g/kg)	100, 75, 50, 25, 10
Switch	cyprodinil (375 g/kg) + fludioxonil (250 g/kg)	10, 5, 1, 0.5, 0.1
Systhane 20 EW	myclobutanil (200 g/L)	50, 30, 16, 6, 3, 0.6, 0.3, 0.06
Teldor	fenhexamid (500 g/L)	100, 75, 50, 25, 10

Photometric testing

The effect of fungicides on spore germination was determined using a photometric method adapted from Pijls *et al*. (1994), which used optical densitometry to measure levels of spore germination. Fungicides were tested at concentrations of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 ppm with three replicates and controls.

Dilutions of each fungicide were prepared in GPM and 100 µl of each pippetted into wells of a flat-bottomed microtitre plate (96 well). 100 µl of non-amended GPM was used as the control. 150 µl of a conidial suspension (at 10^4 spores mL^{-1}) was pippetted in each treatment well and 150 µl GPM only into control wells. Plate absorbance was read at 405 nm immediately after the addition of spores and then 5 days later. The difference in absorbance readings was used to calculate the EC_{50} values for each product/isolate combination.

3.2. Establish the EC_{50} value and lowest concentration of Serenade that produces significant control of *P. ribicola.*

The efficacy of Serenade (a suspension concentrate containing 13.96 g/L *Bacillus subtilis* strain QST 713 at a minimum of $1x10^{12}$ cfu/L) was assessed against three *Phomopsis ribicola* cultures isolated from infected blackcurrant stems. Each isolate was tested using an amended agar test, with Serenade added to PDA at 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001, 0.0000001, 0.00000001, 0.000000001, and 0 ml/L agar. Serenade was added to molten PDA (approximately 80°C) in order to kill the bacteria but not affect the heat stable metabolites which would remain active. Three replicate plates for each isolate and Serenade concentration were inoculated with a 5 mm agar plug taken from the leading edge of a 7 day old culture of the isolate. Colony growth was measured after 7 days incubation at 20°C and an EC_{50} concentration calculated.

Results and Discussion

1. Detection of, and infection criteria for, *Phomopsis ribicola*
   1.1. Optimisation and validation of a *Phomopsis* genus specific antibody for use in ELISA for the detection of *P. ribicola* from blackcurrant plant material.

Results from the tests to determine the correct buffer for extraction of the *P. ribicola* antigen from woody stem material, the optimal dilution rate for use of the Phomopsis antibody and the correct anti-species antibody to use are shown in Table 3. When interpreting results, a 10 fold difference in absorbance between the negative control and the test result was regarded as positive.

Averaging across all results, absorbances of 0.26 and 0.82 were calculated for the mouse and rabbit anti-species antibodies respectively. Comparing these to the negative controls (0.087 and 0.065 for the mouse and rabbit anti-species antibodies respectively) suggested that the Phomopsis antibody had been raised in rabbit and it was this anti-species antibody that should be used in all ELISA testing.

Absorbance results for the three different extraction buffers indicated that the use of coating buffer resulted in the best overall absorbance, with the optimal dilution of the Phomopsis antibody being 1:1000. The absorbance reading obtained for the 1:100 dilution was higher

than the 1:1000, however the 1:100 resulted in higher levels of non-specific absorbance (results not shown) and would also be less cost effective.

Table 3. Absorbance values using the Phomopsis-IgG 59/II ELISA assay and inoculated stem material compared to a negative control

Extraction buffer	Dilution rate of Phomopsis antibody	Absorbance value at 405nm	
		Mouse anti-species Ab	Rabbit anti-species Ab
PBS	1:100	0.111	1.502
	1:1000	0.123	0.288
	1:2000	0.113	0.132
Coating buffer	1:100	0.518	2.059
	1:1000	0.617	0.845
	1:2000	0.623	0.492
Acetic acid	1:100	0.080	1.554
	1:1000	0.092	0.415
	1:2000	0.064	0.091
Negative Control	-	0.087	0.065

Further evaluation of the ELISA, using blackcurrant stem material used as baits failed to give a positive result for *P. ribicola,* even at the highest spore concentration tested (1,000,000 pycnidia). However, repeated negative results for the positive control (extract from wounded inoculated stem material) suggested that the failure may be due to an inactivation of the Phomopsis-IgG 59/II antibody due to repeated freeze/thaw during testing.

1.2. Establish reliable methods for the detection of *P. ribicola* from water and soil samples.

Three different bait types, leaf, green stem and woody stem, were tested for their suitability to detect *P. ribicola* from both water and soil samples. Initially tests were carried out in sterile medium primed with differing spore levels; this provided an indication of how well the bait material could detect *P. ribicola*. The experiment was then repeated in non-sterile media to determine whether the baiting material picked up other fungal species, and if so whether *P. ribicola* could still be detected.

All three bait materials detected *P. ribicola* from both sterile water and sterile soil inoculated with *P. ribicola*. *P. ribicola* was baited more readily from sterile water, with a lower detection

limit of 10 spores/200 ml water compared to 1 spore/ 75 g soil (Table 4). Overall, woody stem material was the most effective bait (Figure 1) with a detection limit of 100 spores/ 75 g soil and 10 spores/ 200 ml water. This compared to limits for leaf and green stem of 10,000 spores/ 75 g soil and, 10 and 100 spores respectively/ 200 ml water.

Table 4. Number of positive bait pieces (out of 4) detected from sterile soil and water inoculated with *P. ribicola* conidial spores

Total No. of spores	Number of bait pieces positive for *Phomopsis ribicola* (max 4)					
	Soil			Water		
	Woody stem	Green Stem	Leaves	Woody stem	Green stem	Leaves
0	0	0	0	0	0	0
10	0	0	0	1	0	2
100	1	0	0	4	4	4
1000	4	0	0	4	4	4
10,000	4	4	4	4	4	4
100,000	4	4	4	4	4	4
1,000,000	4	4	4	4	4	4

Figure 1. Growth of *Phomopsis ribicola* from blackcurrant woody stem material.

When the test was repeated using non-sterile soil or rainwater the presence of faster growing fungal species, such as *Fusarium* spp., meant that the presence or absence of *P. ribicola* in the bait could not be confirmed. The use of the ELISA method to confirm the presence of *P. ribicola* in the bait material was also inconclusive.

The work carried out in sterile substrate inoculated with *P. ribicola* indicated the potential for the use of blackcurrant plant material, in particular woody stems material, as a bait for the detection of *P. ribicola*. However the relatively slow growth of the culture meant that one in a non-sterile culture *P. ribicola* was out-grown, making it impossible to detect on agar plates. There are two potential ways to overcome this, firstly by plating the bait material onto a selective agar, which would allow *P. ribicola* to out-compete the other fungi present or secondly, through the production of PCR primers specific to *P. ribicola*. A literature search indicated that there were no selective agars available for *Phomopsis* species and so one would have to be produced. The production and use of primers would allow detection of the *P. ribicola* DNA in the bait material or direct from the substrate. The added benefit of PCR is that the level of *P. ribicola* present in the substrate could be quantified.

2. Investigation of the infection process

    2.1. Development of methodologies for infecting blackcurrant stems with *P. ribicola*.

Experiments were carried out to determine the optimum method for infecting blackcurrant stems with *P. ribicola*. This would enable controlled disease outbreaks to be introduced into blackcurrant bushes to monitor the factors required for disease spread and also to allow experiments to be undertaken to determine optimum temperatures for infection and expansion of disease symptoms.

Both wounded and unwounded blackcurrant stem sections were inoculated using either a mycelial plug or spore suspension of *P. ribicola*. Both inoculation methods produced disease symptoms on the wounded stems after 14 days incubation at 20ºC. The symptom observed following inoculation with the spore suspension was three times larger than the one following inoculation with a mycelial plug. Observation of the spores after application revealed that the 20 µl inoculum applied to stems was not visible on the wounded stems within a few hours but was visible on the unwounded stems for the duration of the experiment. This suggested that the inoculum on the wounded stems had been absorbed into the stems, with spores distributed within the stem tissue before germination and infection. This is in contrast to the mycelium inoculation which would have taken time to grow into the wound before infection could occur. Continued incubation of the infected

stems led to the production of the long-necked fruiting bodies associated with *Diaporthe strumella,* the sexual state of *P. ribicola* (Figure 2). This capability of the pathogen to sporulate on woody stems under standard conditions indicates that crop debris left on the soil surface of blackcurrant plantations could easily become colonised by *P. ribicola* present in the soil and provide a source of inoculum for subsequent infection of a crop.

No symptoms were found on any unwounded stems, irrespective of the inoculation method, even after three months incubation. This suggested that damage to blackcurrant stems was required for infection by *P. ribicola* to occur. This fits with observation that blackcurrant varieties which are less prone to damage, particularly during the harvesting of blackcurrants are also less prone to stem dieback caused by *P. ribicola*.

Figure 2. Perithecia or long-necked fruiting bodies (arrowed) associated with *Diaporthe strumella,* the sexual state of *Phomopsis ribicola,* found on stem sections inoculated with a conidial suspension of *P. ribicola*.

2.2. Laboratory experiments to determine the optimum temperature for infection, and subsequent symptom development, of blackcurrants stems by *P. ribicola.*

Optimum infection temperature

To investigate the optimum temperature for infection, wounded stems were inoculated using a mycelial plug.

No infection occurred following a 2 hour exposure of wounded blackcurrant stems to *P. ribicola* at any of the temperatures tested (Table 5). Equally there was no consistent infection at 0 and 5ºC at any of the exposures times tested. Infection rates were also inconsistent for all temperatures where the exposure to inoculum was less than 4 days. At temperatures of 20ºC or higher infections occurred after a 1 day exposure to inoculum; however the level of infection was sporadic until an exposure time of 5 days was reached.

Results reported above indicate that infection through a mycelial inoculum will take longer than an infection through spores, as it takes time for mycelia to penetrate far enough into the stem for an infection to occur. As indicated in the previous section, when wounded stems were inoculated with a spore suspension the inoculum was absorbed by the stem within a few hours. This may suggest that under natural conditions the time for infection will depend on whether spores are splash dispersed or wind dispersed. If splash dispersed then the spores are likely to be absorbed by a wound, allowing infection to occur within a few hours.

Table 5. Effect of temperature and time of exposure to inoculum on infection of wounded blackcurrant stem sections by *Phomopsis ribicola*.

Temperature (°C)	**Level of stem infection (%) for a given inoculum exposure inoculum (hours/days)**						
	2 hrs	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**
0	0	0	0	25	0	0	0
5	0	0	0	0	25	0	0
10	0	25	0	25	25	25	50
15	0	0	0	0	0	75	75
20	0	25	100	50	0	75	100
25	0	50	0	25	50	50	50
30	0	25	0	75	0	50	100

Effect of temperature on symptom development

The effect of temperature on symptom extension was tested at temperatures between 0ºC and 30ºC. Combining temperature data to look at the overall effect of incubation time on lesion extension (Figure 3) showed that there was no significant increase in lesion size up to 5 days incubation. However, significant lesion extension was measured following 7 days incubation. Combining incubation time data to show the overall effect of temperature (Figure 4) revealed no significant difference in lesion size by the end of the experiment for temperatures of 10ºC and below. The optimum temperature for lesion growth was 20ºC with the lesion size significantly lower at 15, 25 and 30ºC. The size of lesion at 30ºC was significantly smaller than those at 25ºC.

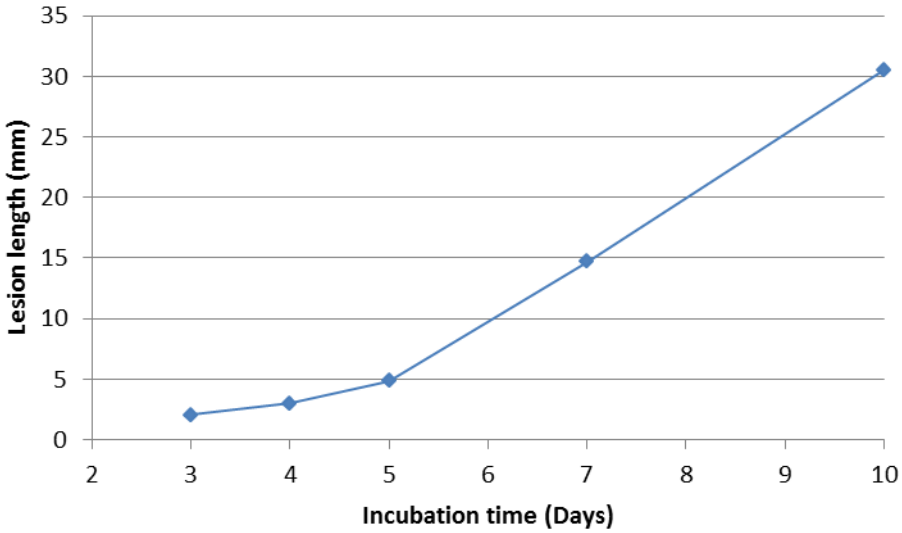

Figure 3. The effect of incubation time on lesion size caused by *Phomopsis ribicola* inoculated onto stem sections of the blackcurrant variety Ben Avon (LSD (5%) = 2.7).

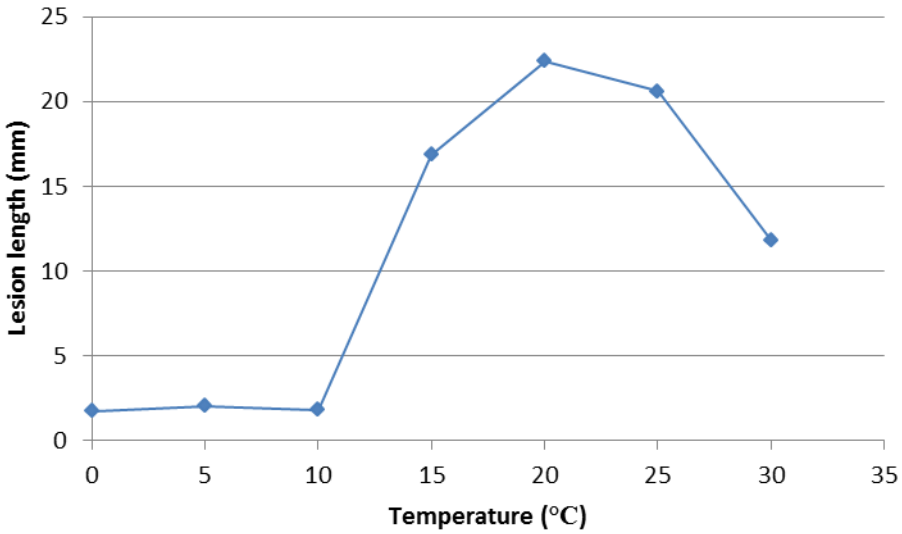

Figure 4. The effect of temperature (ºC) on lesion size caused by *Phomopsis ribicola* inoculated onto stem sections of the blackcurrant variety Ben Avon (LSD (5%) = 3.2).

Both temperature and incubation time had an effect on lesion expansion rate (Figure 5). A small increase in lesion size was seen for temperatures 10ºC and lower, a total increase of 1 to 1.4mm over the 10 day incubation period. For the remaining temperatures tested, there was no significant difference in lesion size up to 5 days incubation. After seven days incubation the lesion size on stems incubated at either 15 or 30ºC was significantly smaller than those incubated at either 20 or 25ºC. Although the lesion size was larger at 15ºC than 30ºC there was no significant difference between the two. After 10 days incubation, the lesion size at 20ºC was significantly larger than the other temperatures and, although not significant, the lesion at 15ºC was larger than those at 25ºC, suggesting that the higher temperature was starting to have a detrimental effect on *P. ribicola* in the stem tissue.

These data suggest that expansion of lesions in infected blackcurrant stem tissue will be relatively rapid at temperatures typical of late spring, summer and early autumn in the UK. The expansion is likely to continue, although at a much reduced rate at temperatures more representative of early spring and late autumn. These laboratory tests were carried out on woody stem tissue and the lesions will be typical of the Phomopsis dieback infections on individual blackcurrant stems. It is likely that where infection occurs at the crown, lesion expansion will be slower due to the denser nature of the tissue.

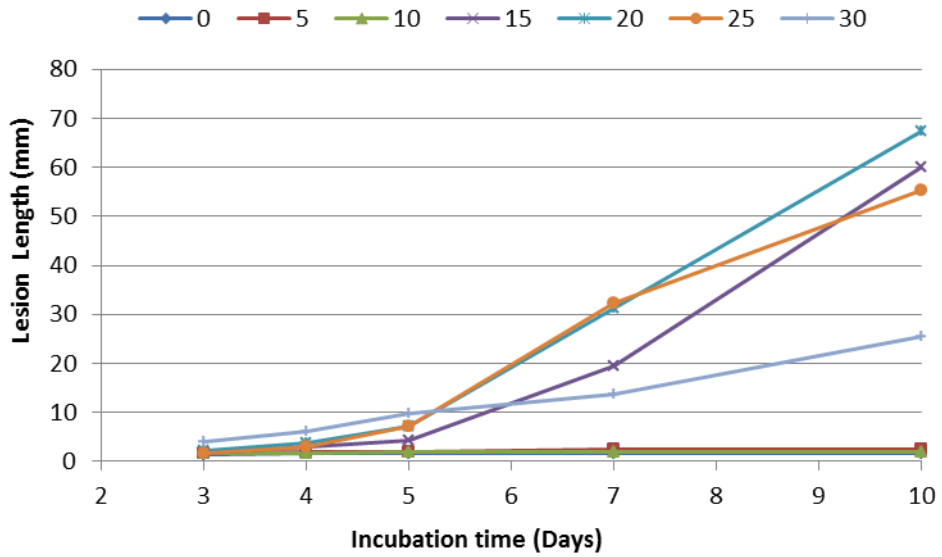

Figure 5. The effect of temperature (ºC) and incubation time on lesion size caused by *Phomopsis ribicola* inoculated onto stem sections of the blackcurrant variety Ben Avon (LSD (5%) = 7.2).

3. Control

3.1. Determine the effect of selected fungicides on the potential biocontrol agent *Bloxamia leucophthalma.*

B. leucophthalma is a fungus which has been isolated from blackcurrant plantations and has been shown to have *in vitro* biological activity against *P. ribicola*. As a result it would be beneficial to try and ensure that any fungicide programmes used to control *P. ribicola* on a blackcurrant plantation did not have an adverse effect on *B. leucophthalma*. This section of the project tested the effect of six fungicides commonly used on blackcurrant on mycelial growth and spore germination of *B. leucophthalma*.

Amended agar test

This test was used to examine the effect of fungicides on the mycelial growth of *B. leucophthalma*. The initial growth screen carried out at an agar fungicide concentration of 10 ppm indicated differences in the efficacy of the six products tested (Figure 6), with Switch having the greatest activity against mycelial growth of *B. leucophthalma* and Stroby the least. From these results a range of test concentrations were chosen for each product in order to obtain EC_{50} values (Table 5).

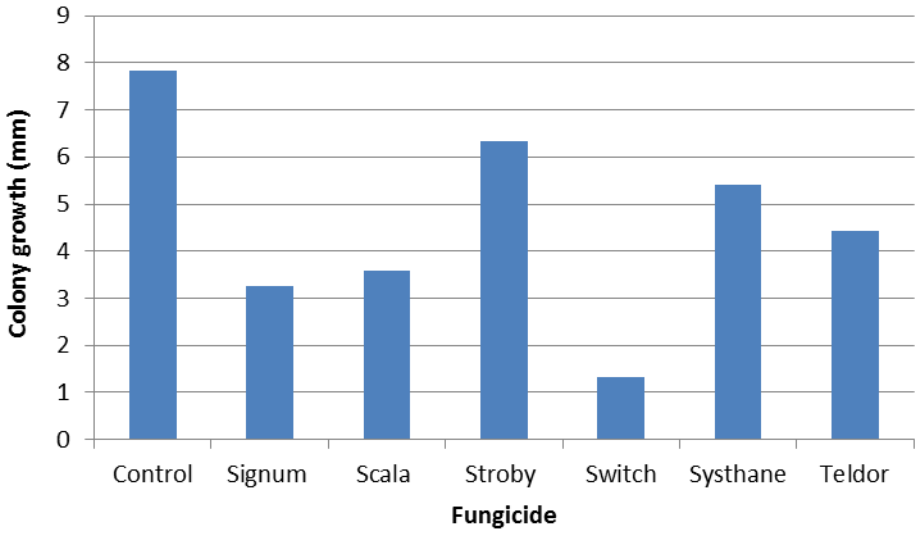

Figure 6. Growth of *Bloxamia leucophthalma* on potato dextrose agar amended to give a total active ingredient concentration of 0 (control) and 10 ppm (test).

The product with the lowest efficacy towards mycelial growth of *B. leucophthalma* and therefore of least impact on the potential biocontrol agent was Stroby (Table 6), with an

EC_{50} greater than 100 ppm (the maximum concentration tested). The next least active products were Teldor and Systhane 20 EW, with EC_{50} values of 54 and 23 ppm of active ingredient respectively. The product with the most activity on mycelial growth of *B. leucophthalma* was Switch, with an EC_{50} value of 0.43 ppm. Results from HDC project SF 12-223 showed that this product was also the most active against isolates of *P. ribicola*.

Table 6. EC_{50} values calculated from an amended agar test for six fungicides tested against *Bloxamia leucophthalma.*

Product	Active Ingredient	EC_{50} (ppm)
Signum	boscalid (267 g/kg) + pyraclostrobin (670 g/kg)	4.85
Scala	pyrimethanil (400 g/L)	5.6
Stroby	kresoxim-methyl (500 g/kg)	>100 (37.6%)
Switch	cyprodinil (375 g/kg) + fludioxonil (250 g/kg)	0.43
Systhane 20 EW	myclobutanil (200 g/L)	23
Teldor	fenhexamid (500 g/L)	54

Photometric test

This test was used to examine the effect of fungicides on spore germination of *B. leucophthalma*. The EC_{50} results from this test followed the same general trend as those from the agar test, with Teldor, Systhane 20 EW and Stroby showing the lowest activity (Table 7). Again, the product with the greatest efficacy against conidial germination was Switch, with an EC_{50} value over 1000 times greater than that of the least active product.

Table 7. EC_{50} values calculated for six fungicides for spore germination of *Bloxamia leucophthalma.*

Product	Active ingredient	EC_{50} value (ppm)
Signum	boscalid (267 g/kg) + pyraclostrobin (670 g/kg)	5.2
Scala	pyrimethanil (400 g/L)	0.56
Stroby	kresoxim-methyl (500 g/kg)	38
Switch	cyprodinil (375 g/kg) + fludioxonil (250 g/kg)	0.09
Systhane 20 EW	myclobutanil (200 g/L)	56
Teldor	fenhexamid (500 g/L)	>100

3.2. Establish the EC_{50} value and lowest concentration of Serenade that produces significant control of *P. ribicola.*

Work carried out in HDC project SF 12-223 showed that Serenade in agar at 10 ml/L gave complete control of *P. ribicola*. In this project, work was undertaken to determine the EC_{50} for Serenade. The results show that Serenade was highly effective at controlling mycelial growth of *P. ribicola* with EC_{50} values ranging from approximately 0.92×10^{-9} to 8×10^{-9} ml Serenade per litre of agar depending on the individual isolate tested (Table 8). The lowest concentration of Serenade that resulted in no growth of the pathogen was 0.001 ml/L.

Table 8. EC_{50} values and growth limiting concentrations for the efficacy of Serenade against mycelia growth of 3 isolates of *Phomopsis ribicola*

Isolate	**EC_{50} value $x10^{-9}$ (ml/L)**	**Growth limiting concentration (ml/L)**
CC48	7.9	0.001
CC79	0.92	0.001
CC2782	7.4	0.001

SF 12-223 concluded that it was toxic metabolites produced by *Bacillus subtilis*, and present in the Serenade formulation, that were active against the pathogen. This conclusion was drawn as no *B. subtilis* colonies were seen growing on the PDA plates, which suggested that the bacteria had been killed when added to molten agar. However, results from the current work showed that colonies of *B. subtilis* grew on plates when Serenade was diluted below 10 ml/L. This suggested that the lack of bacterial growth at Seranade agar concentration of 10 ml/L and above was simply due to an inhibition of growth due to the large number of bacteria involved, not a result of the bacteria being killed when added to agar at 80°C. This was reiterated by the clear serial dilution in bacterial number (Figure 4). The production of growth inhibition zones around single bacterial colonies at dilution rates $1x10^{-7}$ and below (Figure 4) suggested that secondary metabolites produced by *B. subtilis* had an inhibitory effect on the growth of *P. ribicola*.

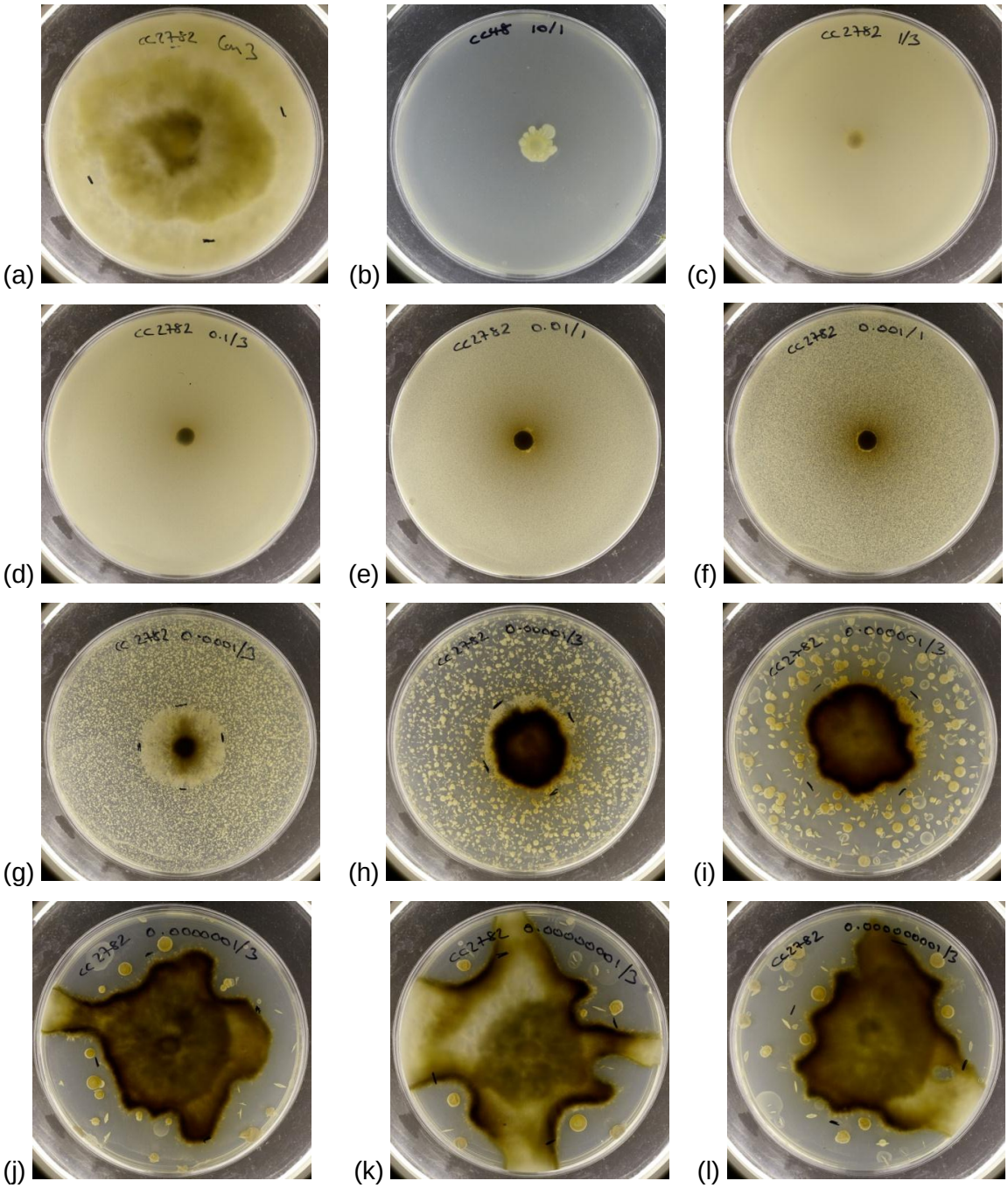

Figure 4. Growth of *Phomopsis ribicola* isolate CC2782 on (a) non-amended agar plates and agar plates amended with Serenade at concentrations ranging from (b) 10 ml/L down to (l) 0.000000001 ml/L.

Conclusions

- Tests using the Phomopsis-IgG 59/II antibody in an ELISA test for the detection of *P. ribicola* in plant material were inconsistent. Production of PCR primers would provide a more consistent and specific method of detection.
- *P. ribicola* was best detected from soil and water using a bait of woody stem material; however a selective agar would need to be developed in order to prevent faster growing fungi from masking its presence during isolation.
- Damage to blackcurrant stems was required for infection to occur. Infection of damaged stems occurred following inoculation with either mycelia or spores.
- Consistent infection of woody stem material occurred at temperatures between 20 and 30ºC after a 5 day exposure to a mycelial inoculum. Infections occurred at temperatures lower than 20ºC although the time required for infection to occur was not consistent.
- Expansion of lesions caused by *P. ribicola* in blackcurrant stems is likely to be relatively rapid at temperatures typical of late spring, summer and early autumn in the UK. The expansion is likely to continue, although at a much reduced rate at temperatures representative of early spring and late autumn.
- It is likely that where infection occurs in the crown then lesion expansion will be slower due to the 'denser nature of the tissue.
- The fungicides Stroby, Teldor and Systhane 20 EW had the least activity against the potential biocontrol agent *B. leucophthalma.* Switch, Scala and Signum had the greatest activity.
- The minimum inhibitory concentration of Serenade towards mycelial growth of *P. ribicola* was 0.001 ml/L, with EC_{50} values ranging from 0.92×10^{-9} to 8×10^{-9} ml/L.The inhibition appeared to result from secondary metabolites produced by living *B*. *subtilis*.

Glossary

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.

Isolate: a pure culture of a micro-organism.

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.

Knowledge and Technology Transfer

N/A

References

Pijls CFN, Shaw MW, Parker A (1994). A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol using a microtitre plate reader. *Plant Pathology*, **43**, 726-32