Project title:	Cucumber: Development and testing of a lateral flow device for gummy stem blight in bio-aerosols during cucurbit production
Project number:	**CP 137a**
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Report:	Final report, December 2019
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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.

Professor Roy Kennedy
Director of Research
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Signature .... Date 15 April 2020 ..

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Signature …….. ..... Date 20 April 2020

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Grower Summary

GROWER SUMMARY

Headline

Diagnostic techniques have been developed to monitor glasshouse air samples for spores which spread ‘Myco’ disease and cucumber powdery mildew. A stable ten minute on-site tests has been developed for Myco inoculum in air samples. Isolates of Myco tested for sensitivity to different fungicide actives showed different levels of sensitivity to boscalid, azoxystrobin and fluxapyroxad. There was no evidence of genetic variation in isolates of Myco collected during different cucumber cropping periods from different production areas.

Background

Improved control of *Mycosphaerella melonis* (Myco), using tests to monitor glasshouse air samples for Myco spore presence were developed in previous projects (CP 137). However these were not stable enough for general use by end users in glasshouse production of cucumbers. With knowledge of ‘Myco’ inoculum, this current project (CP 137a) aims to add to previous work and improve information on timing for fungicide application. The time between finding Myco in air samples and symptom development on a cucumber crop is between two to six weeks. The time period will vary depending on the environment and if control treatments are applied. This means that application of fungicides can be applied before symptoms occur.

*Mycosphaerella melonis (*also known as Black stem rot or Gummy stem blight) is the causative agent of ‘Myco’ on cucumber (syn. *Didymella bryoniae* – sexual stage*).* The disease is of worldwide importance, with significant economic damage of cucurbit crops. The pathogen causes extensive stem & leaf infections which when severe can debilitate or even kill plants. As with the powdery mildew pathogen, airborne spores are produced and are involved in the spread of the disease. The infection of flowers and developing fruit leads to fruit rot, though in many cases disease symptoms are not visible until the fruit is marketed. This leads to rejections and reduced retailer and consumer confidence in the product. Fungicides are used routinely in an attempt to suppress the disease and prevent plant and fruit losses. However, these had been found to provide only a partial suppression or reduction of the disease. No resistant cultivars are available and there is a suggestion that mildew tolerant cultivars are more susceptible to Myco.

Numerous vegetable crops are susceptible to powdery mildew, but cucurbits are one group that are severely affected even though many cultivars now have tolerance (but not resistance) to powdery mildew. In the development phase of crop production fungicides are used routinely for control. It is probably the most common, widespread and easily recognizable disease of cucurbits. Like other powdery mildew diseases, its symptoms are characterized by the talcum-like, powdery

fungal growth that develops on top and bottom leaf surfaces, petioles and stems but rarely on fruits. *Podosphaera xanthii* (also known as *P. fusca*) and *Golovinomyces orontii* are the main agents of cucurbit powdery mildew. The disease provides one of the most important limiting factors for cucurbit production worldwide, and in the absence of chemical, biological control or the use of tolerant/resistant varieties, can result in yield reductions as high as 40%. Inappropriate ventilation, reduced light intensity i.e. partial shade and succulent plant tissue promote disease development, with it being spread via spores (conidia) to other plants on air currents. Although favouring dry conditions, spore release (disease dissemination) can occur at a range of humidities and infection can occur without the necessity of a water film on the plant surface. On mainland Europe, *G. orontii* has been reported during the early cucumber cropping season preferring a dry climate, whilst *P. xanthii* dominates during the summer months as humidity is increased.

The pathogen is unable to survive for more than a few days in the absence of a living host. The length of time between infection of the host plant by the spore and symptom appearance can be as short as 7 days but can take longer than this if conditions are below optimum for the infection process. At present, growers only know that powdery mildew is present once symptom development is observed and the pathogen is established within the crop. The application of fungicides is the principle practice in cucumber cropping for mildew control. However, powdery mildew pathogens have a high potential for fungicide resistance and there is a need for control programmes to be less reliant on blanket spray applications. There are new developments with commercially available bio-control products though in general their level of efficacy is not yet up to the standard required for effective control.

Summary

The work within this project reports on the development of a stable lateral flow test based on new antibody fragments (Fabs). *Mycosphaerella* (Myco) isolates have also been collected from cucumber production areas in the UK and tested for fungicide sensitivity and genetic variation.

Pathogen Monitoring in 2019

During 2019 at protected cucumber production sites in the UK, diagnostic tests have been used to estimate Myco spores in daily and weekly collected air samples. Collected air samples have been assessed for spore types which cause powdery mildew. A microtitre immunospore trap

(MTIST) shown in Figure 1 has been used for laboratory testing of samples.

Figure 1. MTIST air sampler with base plate containing 4x8 well microtitre strips.

Using this technology, Myco spores have been identified in UK glasshouse air samples from March / April onwards using microscopy at two sites. Myco spore levels peak in cucumber production at different times. At site A Myco spores were detected in week 12 however no powdery mildew were detected until week 17. Spore warnings were low / moderate during the first crop up to week 19. High spore concentration in the air were identified for the week 25 commencing 17th June 2019. Spore concentrations for both pathogens recorded in the crop remained high / moderate risk until week 32 commencing 5th August 2019 when the third crop was planted. The levels of powdery mildew recorded were higher than those observed for Myco. Spore concentrations for both pathogens recorded in the crop were highest during weeks 26 27 and 28 before falling to zero as the new crop was planted at that time at site B. The levels of powdery mildew recorded were higher than those observed for Myco. Very high concentrations of powdery mildew were observed from week 35 (August 2019) until the final cropping week. Myco spore levels also increased after week 39 (September 2019). Tube based samplers were also used in monitoring in crop each site. However many of these tubes were used in the development of the Fab based lateral flow tests.

Fungicide Sensitivity Testing

Thirty six isolates were successfully cultured from gummy stem blight infected material collected from eight different commercial cucumber production sites over the course of the 2019 growing season. The results show a considerable degree of variability between isolates to the tested chemicals. It is not clear if this is based on some degree of resistance or just natural variability between isolates. Of the chemicals tested Plover (difenoconazole) was the most effect at reducing Myco growth in agar. Very small amounts of the active ingredient were required to produce a complete control effect. proquinazid and azoxystrobin also showed good control of isolate growth

at the range of concentrations tested. There appeared to be variation in isolate sensitivity to some active ingredients. boscalid, azoxystrobin and fluxapyroxad showed varying degrees of isolate variation to the chemical concentrations used. fluxapyroxad in particular showed a wide variation in response between isolates although the chemical was not particularly effective in controlling Myco.

Genetic variation in isolates

The 36 isolates were analysed in this study originated from gummy stem blight infected material collected from eight different commercial cucumber production sites in the UK. Material was collected and at different times throughout the 2019 growing season (Table). Despite this variation in origin all isolates has been shown to be the same species and RAPD group genotype RG1.

Lateral Flow Tests based on new antibodies

A new lateral flow test has been manufactured using new antibodies produced within this project (CP137A). These have been shown to be stable and can detect spores of *Mycosphaerella melonis* (Myco).

Financial Benefits

The main financial benefits will be in the use of these tests to reduce unnecessary crop protection inputs or to apply more timely crop sprays to cucumber cropping systems. Fungicide usage produces unwanted residues, is costly and can be one of the major inputs in crop production after fuel and labour. The availability of fungicidal control products is also being reduced due to increased regulation. Using the lateral flow device the grower/consultant will be able to check for Myco spores in the air and better time the first fungicide application. Targeted application of control measures will help delay the onset of pathogen resistance to fungicides, thus prolonging their useable life. However, savings will be variable from one season to the next and depend on the number of spray applications made to the crop.

Action Points

New tests for Myco require further testing in commercial cucumber production.

Applications of difenoconazole can control Myco very effectively.

- Other chemicals maybe effective on certain isolates of Myco
- Myco tested showed no variation in genetic type.

SCIENCE SECTION – 2019

The ability to monitor pathogens before disease symptoms are visible in a crop offers many advantages for controlling diseases. This approach is possible by identifying pathogenic spores in the air before they infect the crop. With this information growers are able to time sprays more effectively and make informed decisions as to which type of fungicide application to make. The approach has taken off in many cropping systems ranging from *Peronospora destructor* (onion downy mildew), *Mycosphaerella brassicicola* (ringspot), *Pyrenopeziza brassicae* (light leaf spot) in vegetable crops to *Botrytis* and other diseases (*Mucor* and *Rhizopus*) in strawberries and grapevine.

The approach is also being tailored for control of *Mycosphaerella* (Myco) and powdery mildew in glasshouse production systems which should in theory be easier than monitoring under field conditions. AHDB project CP137A was commissioned to find control solutions using this approach and continued a previous project on this area (CP137). In the previous project antibodies had been produced which recognised *Mycosphaerella melonis* (Myco). However these were not stable enough for commercial usage. Often disease symptoms caused by this pathogen are not visible until the fruit is marketed. This leads to rejection and reduced retailer and consumer confidence in the product. Fungicides are used routinely in an attempt to suppress these diseases and prevent plant and fruit losses. No Myco resistant cultivars are available and mildew tolerant cultivars maybe more susceptible to *Mycosphaerella*. In this report, we describe project activities carried out between November 2018 and November 2019 where new antibodies were raised and assessed for activity within an LFD format. Both Myco and powdery mildew were monitored throughout the season at two commercial sites. Myco isolates were also assessed for genetic variability and sensitivity to a range of fungicidal active ingredients.

Monitoring cucumber pathogens at commercial sites in 2019 using diagnostic probes

Introduction

Air samplers were set up at two commercial cucumber production sites at the beginning of the 2015 growing season. This approach forms the basis of pre-symptomatic monitoring of diseases in crop growing systems. A detailed description of the air samplers, operation and spore assessment is described below (see Methods).

Methods

Weekly bio-aerosol monitoring at a commercial cucumber sites

Air samplers were set up at two commercial cucumber production sites at the beginning of the 2017 growing season. A detailed description of the air samplers, operation and spore assessment is described previously (see CP 137 year 2015 and 2016 https://ahdb.org.uk/cp-137-cucumber-development-and-testing-of-a-lateral-flow-device-for-both-gummy-stem-blight-and-powdery-mildew-in-bio-aerosols-during-cucurbit-production). Samplers consisted of the following types.

Microtitre Immunospore Trap (MTIST). A detailed description of the MTIST device can be found in Kennedy *et al.,* 2000. The sampler contains four microtitre strips each containing 8 wells (Figure 1). The MTIST air sampler uses a suction system and particulates in the airstream are impacted on the base of each collection well of the four microtitre strips. The eight well microtitre strips were coated with a combination of 2 strips at 0.1mg ml-1 Poly-L-Lysine (Sigma P-1524) in distilled water and 0.05% sodium azide (Sigma P-1524) and 2 strips 5:1 mixture of petroleum jelly and paraffin wax (Wakeham *et al.,* 2004).

Burkard cyclone air samplers (**Multivial**). The characteristics of a cyclone air samplers are described by Ogawa & English (1955). Air is drawn through the sampler using a vacuum pump in the form of a cyclone. The height of the cyclone and air inlet, along with the width of the air inlet, air exhaust diameter and the diameter of the cyclone within the length of the exhaust pipe influence the relative efficiency of the trap. These characteristics have been drawn together and standardised within the Burkard cyclone sampler (Burkard Manufacturing Co.). The cyclone air samplers operate at an air flow rate of 10 to 15 L air / min, and air particulates are trapped in a 1.5ml microfuge tube. At each of the sites the sample tube were changed weekly, posted to Warwickshire Colleges and on receipt stored at -20^{0}C. The tubes were used to assess a lateral flow testing device for *M. melonis*.

Results 2019

Objective 1. Monitor *Mycosphaerella and powdery mildew on* commercial crops for spray application timings and to obtain samples for diagnostic testing.

1.1. Establish two commercial sites for monitoring cucumber diseases.

1.2. Complete monitoring of *Mycosphaerella* and powdery mildew at two sites weekly.

Site A

The first crop was removed in week 19 (May 2019) and replanted. The second crop was removed in week 32 (August 2019) and replanted. Myco spores were detected in week 12 however no powdery mildew were detected until week 17. Spore warnings were low / moderate during the first crop up to week 19 (Figure 2). High spore concentration in the air were identified for the week 25 commencing 17th June 2019. Spore concentrations for both pathogens recorded in the crop remained high / moderate risk until week 32 commencing 5th August 2019 when the third crop was planted. The levels of powdery mildew recorded were higher than those observed for Myco. Very high concentrations of Myco were observed from week 36 (September 2019) until the final cropping week. Numbers of powdery mildew spores were lower but significant during that period. Most sprays against Myco and powdery mildew were applied to the second crop although a spray of Signum (boscalid) was applied in week 13. Sprays on the second crop were applied as Reflect (isopyrazam) at week 23 and Biogard (Aq10) applied in the following week. Switch (cyprodinil and fludioxonil) and Amistar (azoxystrobin) were applied to the third crop in week 33. Further applications of Switch and Reflect were applied during week 35 and 36 to the third crop. No further applications of fungicides were applied after that time.

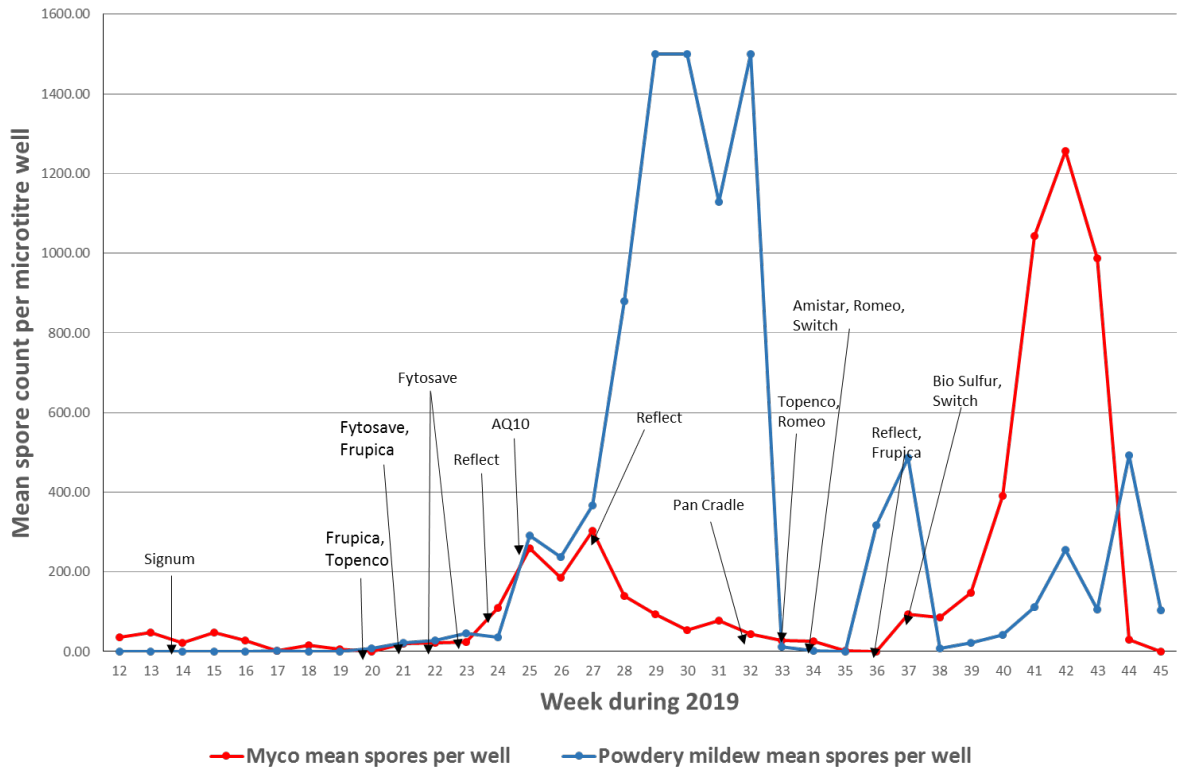

Figure 2 Myco and powdery mildew spore counts with spray applications during 2019 at Site A.

Site B

The first crop was removed in week 18 (May 2019) and replanted. The second crop was removed in week 28 (July 2019) and replanted. Myco spores were detected in week 22 however powdery mildew were detected in week 17 and 18 when the new crop was planted. Spore warning were low / moderate during the first crop up to week 18 (Figure 3). Higher Myco spore concentration in the air were identified for the weeks 24 and 25 (June 2019).

Spore concentrations for both pathogens recorded in the crop were highest during weeks 26 27 and 28 before falling to zero as the new crop was planted at that time. The levels of powdery mildew recorded were higher than those observed for Myco. Very high concentrations of powdery mildew were observed from week 35 (August 2019) until the final cropping week. Myco spore levels also increased after week 39 (September 2019).

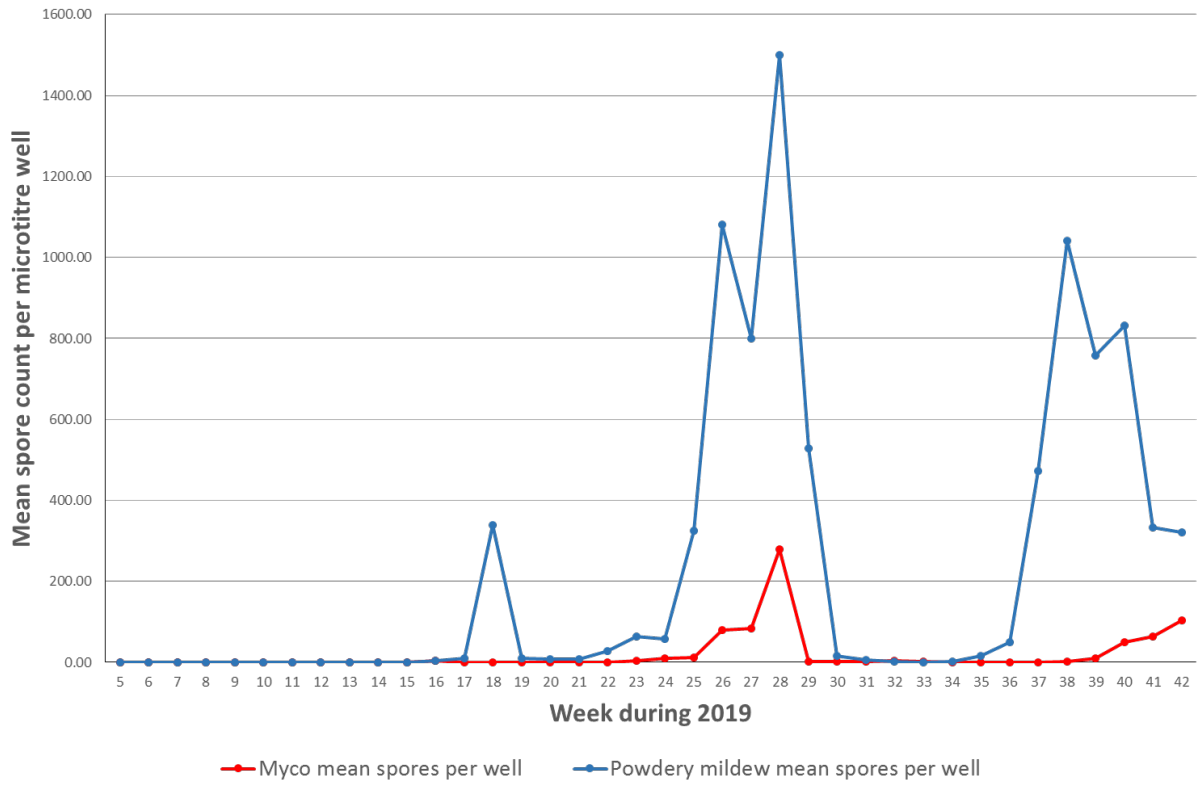

Figure 3 Myco and powdery mildew spore counts during 2019 at Site B.

Conclusions

The results show that both Myco and powdery mildew could be detected in air samples taken in commercial crops well before the onset of symptoms in each crop. At two sites in 2019 *Mycosphaerella melonis* and powdery mildew were both detected during the second cropping period at planting although sporadic numbers of spores were observed during the first cropping period. No data was collected on the infection of the cucumber crop by either pathogen at either site. However the inoculum could have been present on the crop at planting as this was also observed in previous studies. Further information on disease occurrence in these monitored crops and any crop measures applied at site B would be of value. These results and those from the earlier project CP 137 suggest that crops are already contaminated with powdery mildew at planting.

Determining effectiveness of control spray applications in relations to isolates of *Mycosphaerella melonis* from the Humber and Lea valley production areas.

Objective 2. Assess the population of *Mycosphaerella* (Myco) to determine effectiveness of diagnostics and spray applications.

2.1. Complete collection of *Mycosphaerella* (Myco) isolates from all industry areas

Introduction

CP 137 a aims to develop diagnostic assay systems which can be used to monitor glasshouse air samples for gummy stem blight and cucumber powdery mildew. It is expected that these systems will form part of an integrated disease management system and determine when and what type of fungicide application should be made to prevent infection and reduce disease development. However there is little information about the population of *Mycosphaerella melonis* (Myco), which develops on the plant. Objective 2 of this report specifically reports on the population of Myco from different cucumber growing areas. These isolations were examined for their sensitivity to fungicides and any variation in isolate pathogenicity.

Methods

Sample Collection and Isolation from Gummy Stem Blight Infected Material

Infected fruit and stem material were collected from eight different commercial cucumber production sites located in either the East Yorkshire or Essex regions of the UK. Infected material was collected in person or posted to the laboratory by the grower.

Following receipt and logging of samples, isolations were carried out by aseptically removing the hard, outer tissues and soaking small pieces (≤ 5 mm) of infected internal tissue in 1.25% sodium hypochlorite solution for 2 minutes. After three successive washings in sterile distilled water the tissue pieces were placed on potato dextrose agar (PDA) amended with lactic acid (0.4 ml of 25% lactic acid per 100 ml agar). Plates were incubated at 22^{o}C in constant darkness. Following incubation, isolates were sub-cultured on to standard PDA plates before further sub-culture on to PDA slopes under mineral oil for long-term storage.

Results

Thirty six isolates were successfully cultured from gummy stem blight infected material collected from eight different commercial cucumber production sites over the course of the 2019 growing season (Table 1). The commercial production sites were located in the different geographical areas of the UK, East Yorkshire and Essex. Twenty seven of the isolates came from the East Yorkshire sites, nine from the Essex sites and the majority (67%) were taken from the second crop of the season.

All isolates shared a similar morphology, such as colour and rate of growth irrespective of the site they came from (Figure 4). The morphology observed is typical of that of *Didymella bryoniae*.

Isolate reference code	UK County	Commercial Production Site	Date sample material collected	Cucumber crop during 2019
1	East Yorkshire	B	May 2019	1st
2	East Yorkshire	B	May 2019	1st
3	East Yorkshire	B	May 2019	1st
4	East Yorkshire	B	May 2019	1st
5	East Yorkshire	A	July 2019	2nd
6	East Yorkshire	A	July 2019	2nd
7	East Yorkshire	A	July 2019	2nd
8	East Yorkshire	A	July 2019	2nd
9	East Yorkshire	A	July 2019	2nd
10	East Yorkshire	A	July 2019	2nd
11	East Yorkshire	A	July 2019	2nd
12	East Yorkshire	A	July 2019	2nd
13	East Yorkshire	A	July 2019	2nd
14	East Yorkshire	A	July 2019	2nd
15	East Yorkshire	A	July 2019	2nd
16	East Yorkshire	A	July 2019	2nd
17	Essex	D	September 2019	2nd
18	Essex	E	September 2019	2nd
19*	Essex	E	September 2019	2nd
20	Essex	F	September 2019	2nd
21	Essex	G	September 2019	2nd
22	Essex	H	September 2019	2nd
23	Essex	H	September 2019	2nd
24	Essex	H	September 2019	2nd
25	Essex	H	September 2019	2nd
26	East Yorkshire	C	September 2019	2nd
27	East Yorkshire	C	September 2019	2nd
28	East Yorkshire	C	September 2019	2nd
29	East Yorkshire	A	November 2019	3rd
30	East Yorkshire	A	November 2019	3rd
31	East Yorkshire	A	November 2019	3rd
32	East Yorkshire	A	November 2019	3rd
33	East Yorkshire	A	November 2019	3rd
34	East Yorkshire	A	November 2019	3rd
35	East Yorkshire	A	November 2019	3rd
36	East Yorkshire	A	November 2019	3rd

Table 1. Origin of fungi isolated from gummy stem blight infected material from different commercial cucumber growers in the UK

* Isolate 19 was isolated from stem material, all other isolates were isolated from fruit samples.

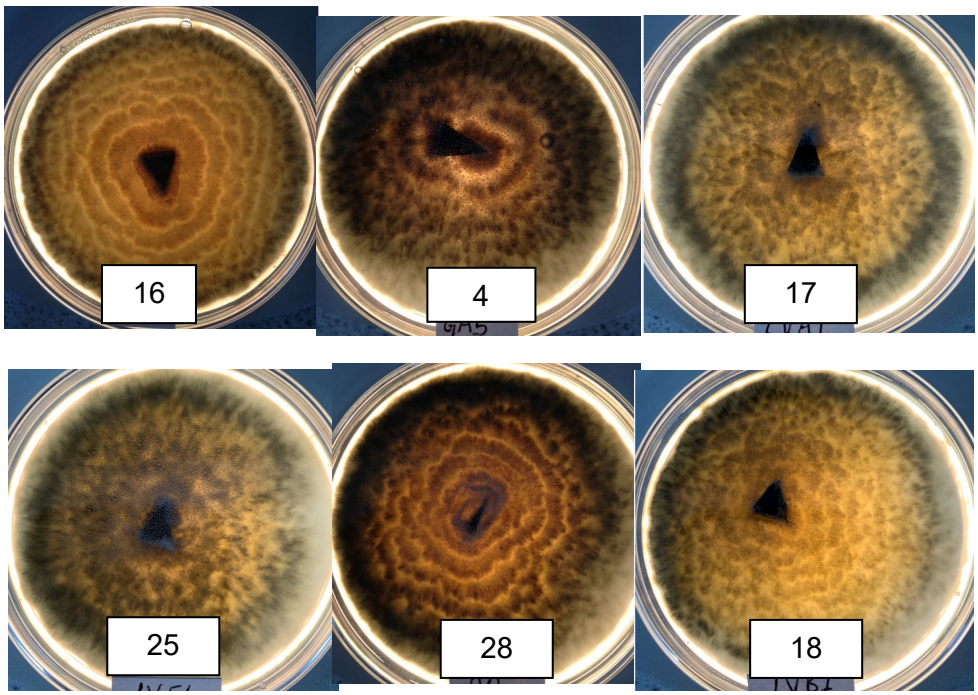

Figure 4. Photographs of 7-day old fungal cultures grown on PDA. All were isolated from gummy stem blight infected fruit or stem material. Pictures are labelled with the isolate's reference code. The culture plates were lit from behind for the photographs.

Conclusions

Isolates of Myco were successfully collected from infected cucumber over all three cropping periods in the Lea valley and Humber production areas. Most isolates were collected from the second cropping period. Only limited numbers of infected cucumbers were available from the first cropping period. Collection of infected cucumbers was delayed in the Lea valley cropping area with the result that only cropping period two had collections organised.

2.2. Obtain DNA extraction from all a sub set of relevant isolates and analyse

Introduction

Morphological characterisation of the isolates of *D. bryoniae* (the sexual stage of *Mycosphaerella melonis* - Myco) and other *Phoma* species from watermelon and other cucurbit crops in the U.S.A. demonstrated variation amongst isolates. Previous studies using RAPD (Random Amplification of Polymorphic DNA) analysis differentiated *D. bryoniae* isolates into three genotypes , RGI, RGII, and RGIV. Other *Phoma* species clustered into a separate though related group RGIII. Sequence characterised amplified region markers (SCAR) based on RAPD profiles have been used for the development of specific PCR primers for differentiation of three *D. bryoniae* groups and the

Phoma group. RGI was the most dominant group in the United States. Isolates of the RGI group were more virulent on watermelon than isolates from other groups.

Methods

DNA Extraction

Seven to 10 day old isolates grown at 22ºC on PDA overlaid with a Supor® membrane (Pall Life Sciences, MI, USA) were taken on for DNA extraction. The membrane was removed aseptically and transferred to the petri dish lid where it was flooded with 5 ml sterile distilled water. Mycelium was removed from the membrane by rubbing with a sterile spreader before transferring to a microcentrifuge tube and centrifuging to pellet the mycelium. DNA was extracted from a small amount (10 to 50 mg of wet weight tissue) of collected material using a commercial DNA extraction kit (FastDNA Spin Kit, MP Biomedicals, Solon USA). Extracted DNA was eluted in molecular grade water and stored at -20ºC (Table 3 gives details of all isolates collected).

Mycosphaerella melonis Presence / Absence PCR

The *Mycosphaerella melonis* (Myco) specific primer sets used here amplify sequences unique to two different *D. bryoniae* genotypes: random amplified polymorphic DNA (RAPD) group I (RG I) and RG II (Somai *et al*., 2002b; Keinath et al., 2003) (Table 2). To determine whether the UK isolates were *D. bryoniae* and to which genotype they belonged they were subjected to a PCR-assay using the RG-specific primers (Somai *et al*., 2002b; Keinath et al., 2003; Babu *et al*., 2015).

For both primer sets the PCR reaction mix contained 25 µl BioMix Red (BioLine Reagents Ltd., UK), 2.5 µl of 10 µM of each primer, 1 µl DNA template and the reaction was brought up to a final volume of 50 µl with molecular grade water. In all cases the DNA template was a 1:100 dilution of genomic DNA in molecular grade water. The PCR amplification was performed in a Bio-Rad T100 Thermal Cycler with the following conditions: 94ºC for 1 min then 30 cycles of 94ºC for 1 min, 64ºC for 1 min and 72ºC for 1 min; followed by 72ºC for 10 mins. The amplified product was analysed on a 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA).

Table 2. Details of primers used for characterisation of gummy stem blight isolates from the East Yorkshire and Essex areas of the UK (Somai *et al*., 2002b; Keinath et al., 2003).

Primers	Sequence (5'-3')
RG I-F	TGTCGTTGACATCATTCCAGC
RG I-R	ACCACTCTGCTTAGTATCTGC
RG II-F	GCTAAGCCTTAATCTAGCTGC
RG II-R	GAGAGTAAGCTAACCTAAAGG

Results

For all 36 isolates, the RGI primer set produced the expected 730-740 bp fragment (Babu *et al*., 2015; Ha *et al*., 2009) after amplification in a PCR assay (Figure 5). The RGII primer set did not amplify DNA for any of the isolates (Table 3). All 36 isolates were identified as *Mycosphaerella melonis*, genotype RGI.

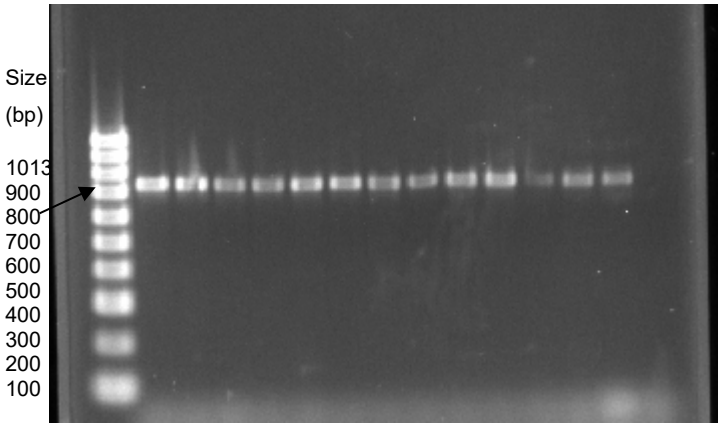

Figure 5. DNA amplified using the RGI primer set. Lane 1: molecular size marker, lanes 2 to 14: isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13; lane 15: H_2O negative control

Table 3. Origin and identification of fungal isolates using the *D. bryoniae* RG-specific primer sets (*D. bryoniae* is equivalent to *Mycosphaerella melonis* in these tests but technically we can only use the nomenclature from the paper)

Isolate reference code	UK County	Commercial Production Site	Crop	RGI primer set	RGII primer set	Identification
1	East Yorkshire	B	1st	+	-	*D. bryoniae*, genotype RGI
2	East Yorkshire	B	1st	+	-	*D. bryoniae*, genotype RGI
3	East Yorkshire	B	1st	+	-	*D. bryoniae*, genotype RGI
4	East Yorkshire	B	1st	+	-	*D. bryoniae*, genotype RGI
5	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
6	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
7	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
8	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
9	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
10	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
11	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
12	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
13	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
14	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
15	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
16	East Yorkshire	A	2nd	+	-	*D. bryoniae*, genotype RGI
17	Essex	D	2nd	+	-	*D. bryoniae*, genotype RGI
18	Essex	E	2nd	+	-	*D. bryoniae*, genotype RGI
19*	Essex	E	2nd	+	-	*D. bryoniae*, genotype RGI
20	Essex	F	2nd	+	-	*D. bryoniae*, genotype RGI
21	Essex	G	2nd	+	-	*D. bryoniae*, genotype RGI
22	Essex	H	2nd	+	-	*D. bryoniae*, genotype RGI
23	Essex	H	2nd	+	-	*D. bryoniae*, genotype RGI
24	Essex	H	2nd	+	-	*D. bryoniae*, genotype RGI
25	Essex	H	2nd	+	-	*D. bryoniae*, genotype RGI
26	East Yorkshire	C	2nd	+	-	*D. bryoniae*, genotype RGI
27	East Yorkshire	C	2nd	+	-	*D. bryoniae*, genotype RGI
28	East Yorkshire	C	2nd	+	-	*D. bryoniae*, genotype RGI
29	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
30	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
31	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
32	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
33	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
34	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
35	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI
36	East Yorkshire	A	3rd	+	-	*D. bryoniae*, genotype RGI

+ Target fragment was amplified during the PCR assay using RG-specific primers.

- DNA was not amplified during the PCR assay using RG-specific primers.

Discussion

In a study by Babu *et al*. (2015), 25 gummy stem blight isolates found to belong to the RAPD group RGI after analysis using the RG-specific primers where sequenced and compared with nucleotide sequences on the National Centre for Biotechnology Information (NCBI) database. All 25 RGI isolates showed 99% identity with a *Stagonosporopsis cucurbitacearum* isolate in the

database. *Didymella bryoniae* is a synonym of *S. cucurbitacearum*. This allows us to be confident that the gummy stem blight isolates collected over the course of the 2019 growing season from eight different commercial cucumber production sites, located in two geographically different areas of the UK, are *Didymella bryoniae* which is the sexual form of Mycosphaerella melonis (Myco).

The dominance of the RGI genotype amongst our isolates is in agreement with other studies that have used the RG-specific primers to identify gummy stem blight isolates from other parts of the world, such as the USA (Babu et al., 2015; Ha et al., 2009; Somai et al., 2002a).

The *D. bryoniae* RGI genotype has previously been found to be more pathogenic than the RGII genotype when tested on watermelons (Somai et al., 2002a), although a more recent study investigating gummy stem blight isolates from USA (Babu et al., 2015) found variability in pathogenicity among their RGI isolates.

The 36 isolates analysed in this study originated from gummy stem blight infected material collected from eight different commercial cucumber production sites, over two geographically different areas of the UK and at different times throughout the 2019 growing season (Table 3). Despite this variation in origin all isolates have been shown to be the same species and RAPD group genotype.

2.3 Test relevant isolates for fungicide sensitivity

Methods

Seven of the isolates (isolates 16, 4 and 28 from East Yorkshire and 17, 18, 19 and 24 from Essex) were tested for their sensitivity to five different fungicides containing active ingredients considered likely to have some level of control of *Mycosphaerella melonis* (Table 4).

Isolates were tested for their sensitivity to the fungicides by measuring mycelial growth on potato dextrose agar (PDA) plates amended with the test chemicals. Seven to 10 day old cultures grown on PDA at 22ºC were used as inoculum for the sensitivity tests. All of the fungicides were tested at 0, 2, 20 and 100 parts per million (ppm) of active ingredient. Plover was additionally tested at the lower concentrations of 0.02, 0.2 and 1 ppm. A stock solution, made from the fungicide but at a 1000 ppm concentration of the active ingredient, was used to achieve the desired concentrations of each fungicide within the PDA. Once inoculated with a 5 mm core of culture, plates were incubated at 22ºC in constant darkness and radial growth was measured after three days incubation. For all isolates, three replicate plates were inoculated at each fungicide concentration and the percentage growth inhibition was calculated based on the 0 ppm control.

Table 4. Fungicides used in the sensitivity tests and active ingredient concentrations

Trade Name	Active Ingredient	Active Ingredient Concentration	Amount to add to 100ml SDW to produce 1000ppm active ingredient stock solution
Amistar	azoxystrobin	250 g/litre	0.4 ml
Filan	boscalid	50% w/w	0.2 g
Plover	difenoconazole	250 g/litre	0.4 ml
Sercadis	fluxapyroxad	300 g /litre	0.33 ml
Talius	proquinazid	200 g/litre	0.5 ml

Results

The results for each chemical test are shown in the Figures below. They are shown for 2, 20 and 100 ppm unless the chemical was particularly effective.

Amistar (azoxystrobin)

Figure 6 shows that an increase in concentration from 2 to 20 ppm corresponds to an increase in growth inhibition, increasing from a mean of 24 % inhibition to 60 %. A reduced effect was observed at 20 ppm to 100 ppm where inhibition only increases by an average of 3 %. Of the seven isolates, one (isolate 18) appears to be more sensitive than the others to Amistar. Isolate 18 was isolated from a cucumber that originated from a commercial grower in the Essex area (Table 1). Growth of isolate 18 was inhibited by more than 50% at the lowest concentration tested, although, as with the other isolates, inhibition increased only slightly at the higher Amistar concentrations, ranging from 67 % at 20 ppm to 70 % at 100 ppm. Mean growth inhibition, using data from all of the isolates tested, at the 100 ppm concentration of Amistar is 63 %.

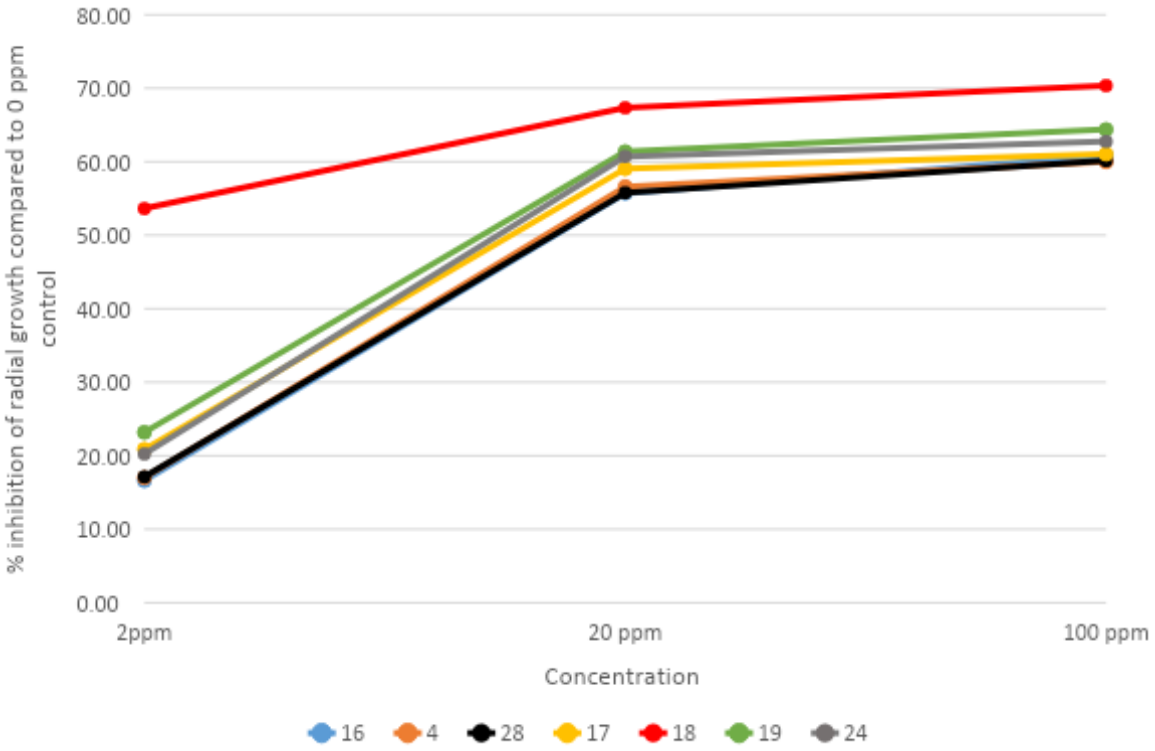

Figure 6. Isolate sensitivity to Amistar (azoxystrobin)

Filan (boscalid)

For five (isolates 16, 4, 19, 24 and 28) out of the seven isolates, Filan failed to inhibit growth by more than 50 % at any of the concentrations tested (Figure 7). Mean inhibition at the highest concentration of 100 ppm for these five isolates was 44%. The overall mean growth inhibition at the 100 ppm concentration, taking into account all isolates, was 52 %. Again isolate 18 showed greatest sensitivity, with an initial inhibition of growth of 71 % at the lowest concentration tested but only rising by a further 10 % with the increase in concentration to 100 ppm. Isolate 17 also proved to be more sensitive to Filan but only at the highest concentration tested. Isolate 17 was also isolated from a cucumber obtained from a commercial grower in the Essex area (Table 1). This isolate was inhibited by only 26 % at the 2 ppm concentration but unlike isolates 16, 4, 19, 24 and 28 it was increasingly inhibited as the concentration of Filan increased, reaching 45% inhibition at 20 ppm and 60% at 100 ppm.

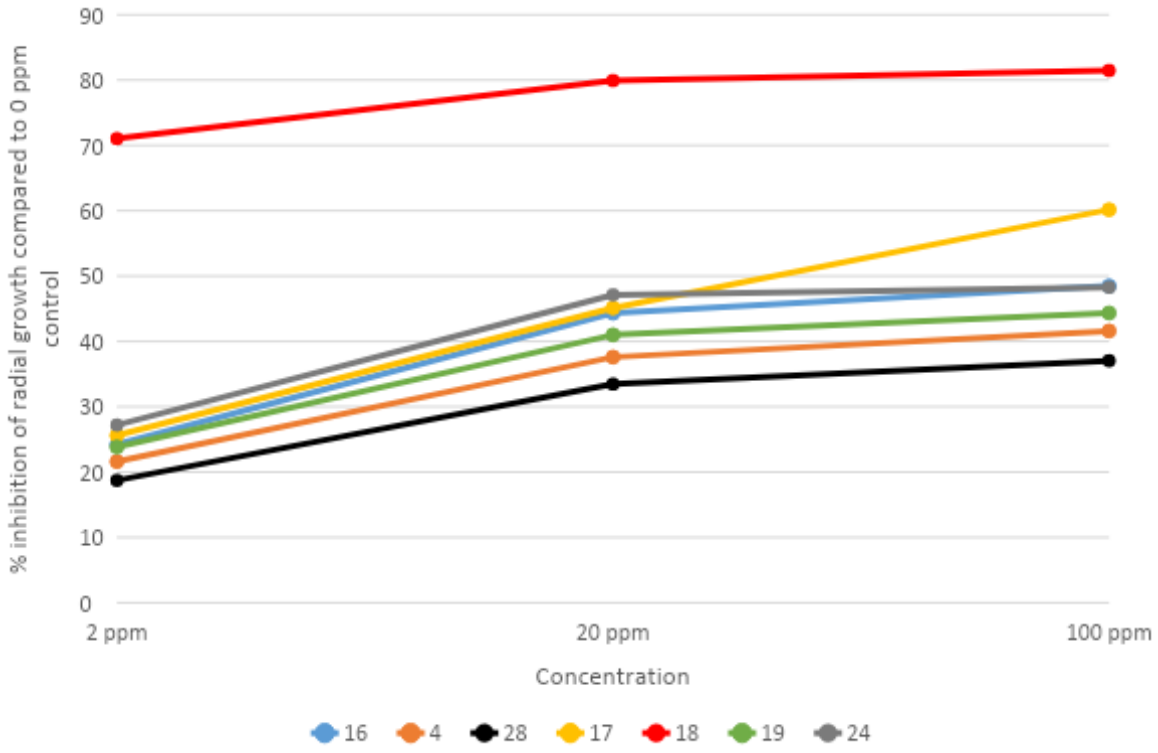

Figure 7. Isolate sensitivity to Filan (boscalid)

Plover (difenoconazole)

Initially, Plover was tested at 2, 20 and 100 ppm, however at all of these concentrations 100% growth inhibition was seen with all isolates (Figure 8). Due to this strong inhibitory effect Plover was again tested but at the lower concentrations of 0.02, 0.2 and 1 ppm. At the lowest concentration tested (0.02 ppm) mean growth inhibition was 60%, rising to a mean of 87% inhibition at 0.2 ppm before complete inhibition of growth of all isolates at 1 ppm. All isolates were affected to a similar degree by Plover, no isolate appeared to be more or less sensitive than the others.

It should be noted that Plover contains 60-70% solvent naphtha (petroleum) which could have had an effect on the growth of the isolates. Further testing would be required to determine whether the solvent did affect growth.

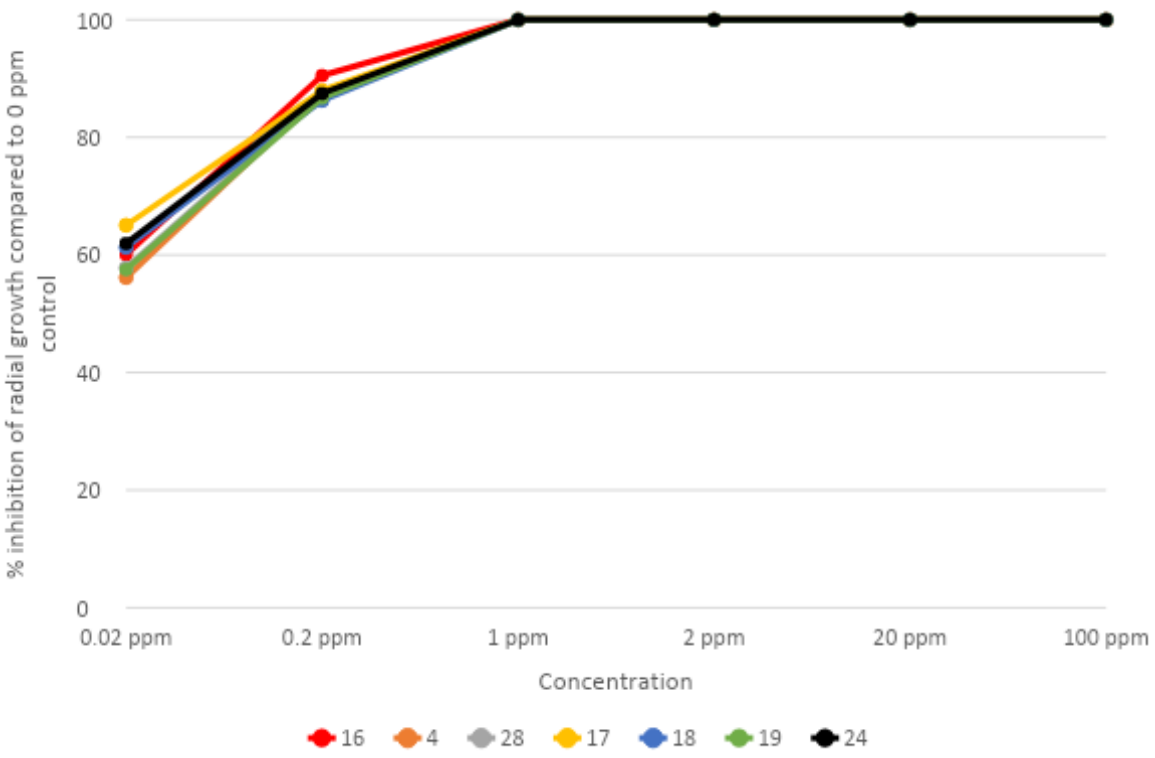

Figure 8. Isolate sensitivity to Plover (difenoconazole)

Sercadis (fluxapyroxad)

The effect of Sercadis was variable amongst the seven isolates, with three distinct groups visible in Figure 9. As with Amistar and Filan, isolate 18 demonstrated greatest sensitivity with a growth inhibition at the lowest concentration tested of 76 % rising to 93 % at 100 ppm. In comparison, the mean inhibition of growth for isolates 16, 17 and 24 at 100 ppm was only 64 %. Mean growth inhibition at 100 ppm for the middle group of isolates visible in Figure 9 (isolates 4, 19 and 28) is 74 %. Overall mean growth inhibition, when including all isolates, at the 100 ppm concentration of Sercadis, was 72%.

Although there are three distinct groups based on percentage growth inhibition, the dose response is similar for all isolates. Only a slight increase in growth inhibition was observed with the increase in concentration of Sercadis. The mean increase in inhibition over the concentration range 2 ppm to 100 ppm was 18 %.

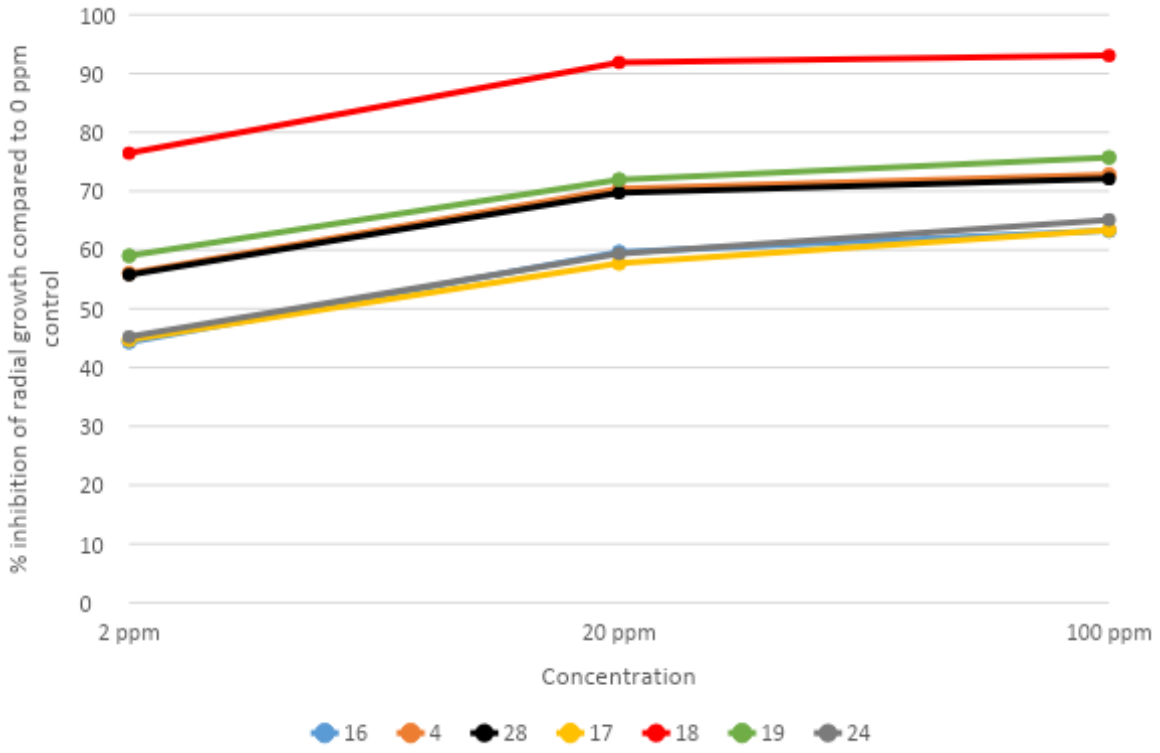

Figure 9. Isolate sensitivity to Sercadis (fluxapyroxad)

Talius (proquinazid)

A dose response is visible in Figure 10 where percentage growth inhibition can be seen to increase with the increase in concentration of Talius. All isolates showed a similar response with inhibition ranging from a mean of 13 % at 2 ppm to 80 % at 100 ppm, an average increase in growth inhibition of 67 % over the concentration range tested.

All isolates were affected to a similar degree by Talius, no isolate appeared to be more or less sensitive than the others.

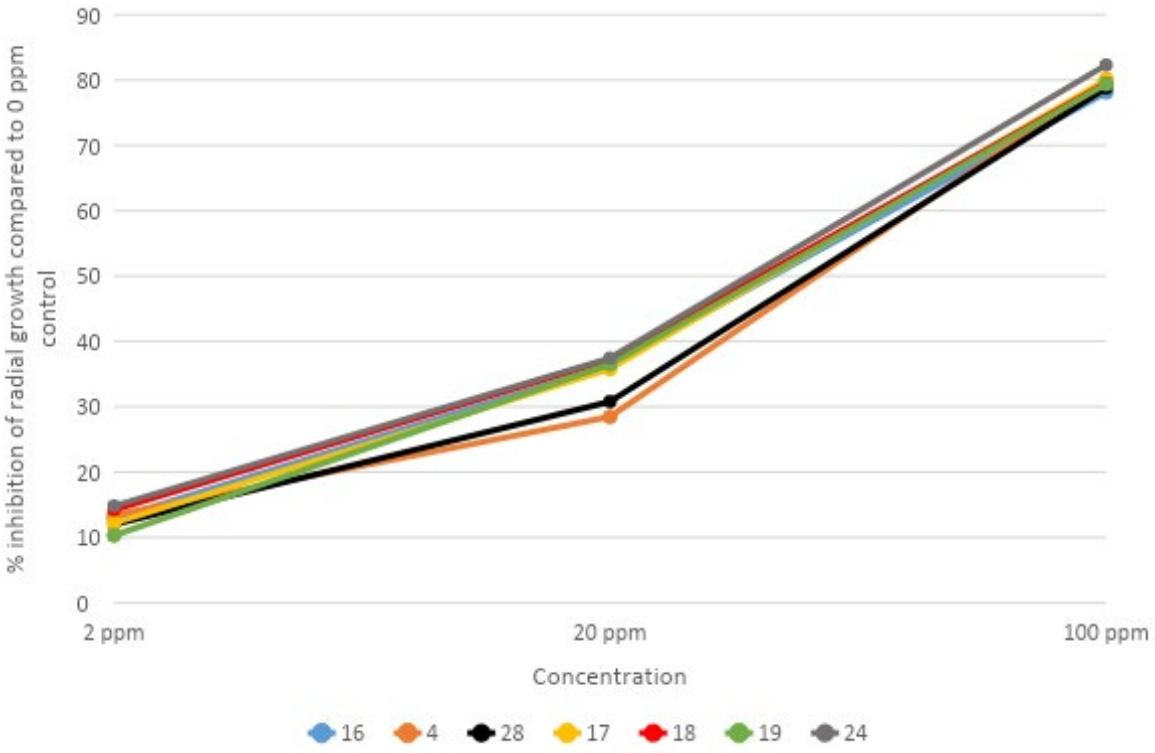

Figure 10. Isolate sensitivity to Talius (proquinazid)

Conclusions

The results show a considerable degree of variability between isolates to the tested chemicals. It is not clear if this is based on some degree of resistance or just natural variability between isolates. Of the chemicals tested Plover (difenoconazole) was the most effect at reducing Myco growth in agar. Very small amounts of the active ingredient were required to produce an effect. Proquinazid and azoxystrobin also showed good control of isolate growth at the range of concentrations tested. There appeared to be variation in isolate sensitivity to some active ingredients. Boscalid, azoxystrobin and fluxapyroxad showed varying degrees of isolate variation to the chemical concentrations used. Fluxapyroxad in particular showed a wide variation in response between isolates although the chemical was not particularly effective in controlling Myco.

Cucumber *Mycosphaerella melonis* (Myco) Lateral flow Development

3. Improve the stability of the *Mycosphaerella* antibody for use in LFD tests and provide an LFD test supply for cucumber growers.

Introduction

Project CP 137 produced a IgM antibody cell line (2B11D1D2) which recognised Myco spores. However this cell line was not stable enough for usage in lateral flow tests. It is hoped that the production of F(ab) antibodies based on this cell line may improve this stability. The F(ab) fragment is an antibody structure that still binds to Myco antigens but part of the original antibody is missing.

To produce this fragment an antibody is digested by the enzyme “papain” yielding two F(ab) fragments of about 50 kDa each and an Fc antibody fragment. Antibody (F(ab')2 fragments) can also be produced by “pepsin” digestion of the whole IgG or IgM antibodies to remove most of the Fc antibody region while leaving intact some of the hinge region. F(ab')2 fragments have two antigen-binding F(ab) portions. Antibody fragments (F(ab) are powerful tools that block background from primary antibody binding and potentially improve stability.

3.1. Complete production of Fab gold conjugation

Methods

The Fab production was required before gold conjugates could be produced.

Production of IgG Fab (murine with His Tag)

Cell line 2B11D1D2 for isolation of Fab sequence data and production of recombinant Fab material was supplied to Mologic from Warwickshire Colleges who carried out the sequencing.

Stage I – Hybridoma sequencing

Hybridoma cells were grown at Warwickshire Colleges. Hybridoma sequencing was performed by whole transcriptome shotgun sequencing. The DNA and protein sequences of the mature variable heavy and variable light regions of the antibody were then identified.

Stage II – Synthesis and cloning

Variable domains were designed and optimised for expression in mammalian cells (HEK293) prior to being synthesised. The sequences were subcloned into an Absolute Antibody cloning and expression vector for the appropriate isotype and subtype of immunoglobulin heavy and light chains.

Stage III – Pilot expression and purification (30 or 50 mg scale)

HEK293 cells were passaged to the optimum stage for transient transfection. Cells were transiently transfected with heavy and light chain expression vectors and cultured for a further 6-14 days. An appropriate volume of cells were transfected with the aim of obtaining 30 or 50 mg of purified antibody.

Cultures will be harvested and a one-step purification performed using affinity chromatography. Upon successful purification the antibodies will be buffer exchanged into the buffer of choice. The antibody will be analysed for purity by SDS-PAGE and concentration determined by UV spectroscopy. If the end use requires low endotoxin levels, such as in vivo studies, all stages of purification will be performed following standard procedures to minimise endotoxin level.

Results

The light and heavy chain sequences for the Fab fragments produced from cell line 2B11D1D2 are shown in Table 5

Table 5 Light (VL) and Heavy (VH) chain Fab fragment sequences produced for cell line 2B11D1D2

ID	Ab Chain	Amino acid Sequence
Clone 2B11D1D2	**Light Chain**	>2B11D1D2_LC_fab DIVMTQSHKFMSTSVGDRVSITCKASQDVGTAVAWYQQKPGQSPKLLIYWASTRHTGVPDRFTGS GSGTDFTLTISNVQSEDLADYFCQQYSSYPLTFGAGTKLELKRADAAPTVSIFPPSSEQLTSGGASVVC FLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKT STSPIVKSFNRNESYPYDVPDYAS
	Heavy Chain	>2B11D1D2_HC_fab EVQLVESGGGLVQPKGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKSNNYATYYADS VKDRFTISRDDSQSMLYLQMNNLKTEDTAMYYCVRRYFDVWGAGTTVTVSSESQSFPNVFPLVSCE SPLSDKNLVAMGCLARDFLPSTISFTWNYQNNTEVIQGIRTFPTLRTGGKYLATSQVLLSPKSILEGSD EYLVCKIHYGGKNRDLHVPIPAVACHHHHHHH

Assessment of reactivity of Fab and IgM antibodies to Myco ascospores by PTA ELISA

The Fab and IgM antibodies derived from cell line 2B11D1D2 were adjusted to 0.1 and 1.0 ug/ml and assessed by Plate trapped antigen enzyme linked immunosorbent assay (PTA ELISA) (protocol supplied by WCG) on control wells (no Myco present) and microtitre MTIST wells (wells containing ascospores of Myco) from cucumber production exposed wells. The wells either had no spores, 500 or 2500 *M. melonis* ascospores trapped on their base.

Results

Both the IgM and the Fab derived cell line (2B11D1D2) responded in a similar manner to the *M. melonis* ascospores trapped on the microtitre base well by PTA ELISA. No reactivity was observed by either antibody for wells containing no Myco spores.

Conclusion

The IgM and the Fab derived cell line recognised the homologous antigen in an ELISA solid phase immunoassay. There was no difference between the signals generated.

Optimisation of buffer pH for gold conjugation of native 2B11 Fab

Method

The native Fab was assessed for conjugation to gold colloid at OD1 across a range of pHs and antibody loadings (Table 6).

Material	**Supplier**	**Product code**
Gold	BBI	EMGC40
CP 137		Native fab 1mg/ml
Conjugation buffers	**Molarity**	**pH**
MES	20mM	5.3
MES	20mM	6.7
TES	20mM	7.1
TAP	20mM	7.4
TES	20mM	7.5
TAPs	20mM	8.5
Borate	20mM	8.5
Borate	20mM	9.0
Borate	20mM	9.0
Borate	20mM	9.3

Table 6 Conjugation buffers used to determine optimal gold conjugation process

Antibody loading

To achieve the specific Fab loading on the gold particle, the Fab was diluted in the chosen buffer and across a concentration range (Table 7). The conjugation loadings were then transferred to a 96 well low bind ELISA plate

Row	Antibody ul	Buffer ul	Loading ug/ml
A	0	30	0
B	4	26	1
C	8	22	2
D	12	18	3
E	16	14	4
F	20	10	5
G	24	6	6
H	0	30	0

Table 7. Amounts of Fab and buffer used in combination for gold conjugation.

Results

At a high F(ab) loading and, with the exception of MES buffer (20Mm), the buffers assessed were only able to stabilise the F(ab) at >pH8.5 (Figure 11). Following addition of NaCl the Fab was not stable under any of the conditions. Conjugate aggregation is indicated by purple colouration of the conjugation. An aggregation ratio of >3.5 is considered optimal for performance within lateral flow assays.

A H

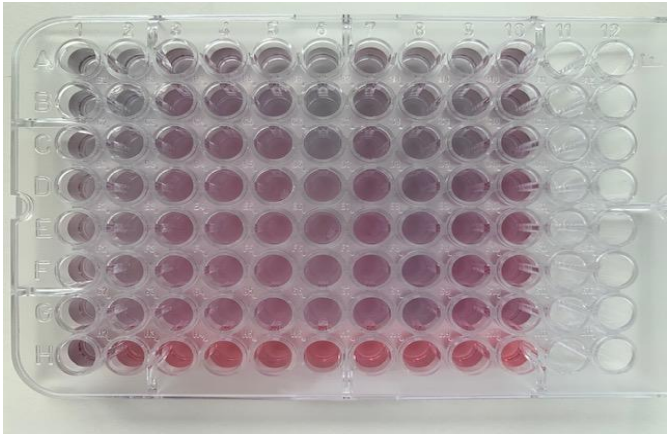

Figure 11 Conjugation aggregation of Fab gold conjugates

Conclusion

The results determine that the Fab in its native state would remain stable in wet assay but was not stable on drying down in 'dry assay state'. It was decided the next step would assess the Fab conjugated directly to BSA and indirectly conjugated to gold at a gold OD5

Optimisation of buffer pH for the conjugation of BSA conjugated 2B11 Fab monoclonal to gold colloid

Method

The Fab conjugated to BSA was assessed for gold conjugation stability with gold colloid at OD1 across a range of pHs at 6ug/ml. For this purpose, 750ul BSA conjugated FAb was centrifuged at 4000g for 10 mins and the supernatant was removed. To inhibit bacterial growth 3.75ul 10% sodium azide was aliquoted to the sample. The Fab was adjusted to 50ug/ml by 1.6ul Fab amended with 48.4ul Buffer (Table 8). A dilution series of each Fab / buffer was prepared as described previously and assessed for stability prior to and after addition of NaCl.

Material	**Supplier**	**Product code**
Gold	BBI	EMGC40
CP 137		BSA fab 1.6mg/ml
NaCl	Fisher	
Conjugation buffers	**Molarity**	**pH**
TAPs	20mM	8.5
Borate	20mM	8.5
Borate	20mM	9.0
Borate	20mM	9.3

Table 8. Materials used for assessment of gold conjugation stability of native 2B11 Fab

Results

Aggregation ratios for BSA Fab showed a similar pattern to those previously observed with the native Fab (Figure 12). However, following addition of BSA at an antibody loading of 6ug/ml a suitable conjugation was observed with a Borate 20Mm buffer (pH 9.3).

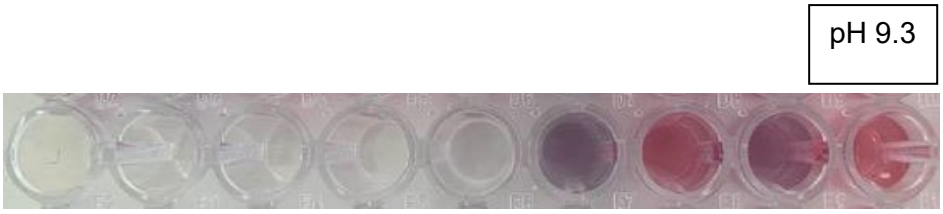

Figure 12 Fab BSA gold conjugation with range of conjugation buffers

Conclusions

It was appropriate to wet lateral flow assay testing with BSA Fab at an antibody gold conjugation loading of 6ug/ml with a Borate 20Mm buffer (pH 9.3).

Wet testing of gold conjugates

In the early stages of development a lateral flow assay is normally tested wet where the conjugate and the sample are mixed together before being added to the strip. Four variants (simultaneous addition, sequential addition, sample first, and Immunoprecipitation) of wet conjugate testing are tested.

Method

A range of lateral flow membranes were striped with *Mycosphaerella* (Myco) antigen to form a homologous test line. Only the optimal process and materials are described (Table 9).

Material	**Supplier**	**Product code**
Antigen	WCG	Myco RS
80mm Backing card	Lohmann,	LC-58384
Sink Pad	Ahlstrom	222 22mm
Sample pad	Whatman	8214 6621
Top laminate (red)	Lohmann	LC58386
Bottom laminate	Kenoshatape	KN-CPP1-OL50

(clear)		
Conjugate pad 27mm	Millipore	8971
NC 40mm	MDI	200 CNPH -N-S560
PBS	Sigma	P4417
Anti-Mouse	Lampire	7455507
50% sucrose PBS	Fisher	S/8600/60

Table 9 Preparations used for test and control line antigen

Test line antigen preparation

The Myco antigen was centrifuged for 5mins at 13,000rpm. The supernatant removed and the pellet discarded. There was no addition of sucrose

Control line material

The anti-mouse was adjusted to 1mg/ml and a ratio of 50ul anti mouse to 10ul 50% sucrose and 440ul PBS was prepared

Nitrocellulose membrane with control and test line antigen application

A test line was plotted at 15mm, 0.1ul/ml, membrane dried at 37ºC, 10mm/sec. Following drying, the test line antigen was plotted again and dried. The test line material was plotted for a 3rd time, on this occasion a control line was plotted at 0.1ul/mm (20mm). The membrane was dried again and stored with desiccant in foil pouches.

Laminating of Nitrocellulose test and control lines

The middle strip of the backing card was removed and placed on to the laminator. The membrane was placed onto the backing card 25mm from the bottom of the card so that the control line is closest to the top of the card. With the card in the laminator the sink pad was positioned so that it butted against the top of the card along its entire length, forming an overlap with top of membrane. The backing card was then removed from the laminator and the sample pad positioned along the bottom edge of the membrane (nearest the test line) to give an approx. 2.5mm overlap. The conjugate pad was positioned on the card so that it was adjacent to the base of the card along its entire length, forming an overlap with the base of the sample pad and membrane. At each operation firm contact was made between all the components and the backing card. From the base of the sink pad, 6mm was measured and marked at each end of the card. The top tape was position so that it is aligned with these marks, and completely covered the sink pad and continued on the back of the backing card Due to variations arising from the folding of laminate over the material junctions a tolerance of +/- 1mm can be expected.

Similarly marks 6mm from the top of the conjugate pad were made to allow positioning of the bottom tape so that it aligned with these marks, and completely covered the conjugate pad and continued on to the back of the backing card. The tape was firmly attached to the band, ensuring that there was no excess tape beyond the backing and that no bubbles were present. To ensure good contact between the different laminate sections a hand-held roller was pressed back and forth, once. This was repeated for the bottom section of the card. Using the Biodot cutter, the laminated card in to 4mm strips. The strips were immediately placed in sealed foil pouch with desiccant and labelled

Results

Both the IgM Myco and the BSA Fab did not bind to the homologous antigen test line.

Conclusion

Both the IgM and the Fab were found to be functionally active to homologous antigen (ascospores of Myco) in PTA ELISA. In this assay format the antibody kinetic dynamic binding range (k on / k off rate) will tolerate an antibody of low affinity with high avidity. These characteristics are associated with IgM antibodies. The recombinant Fab will retain the low affinity characteristics of the IgM hybridoma cell line.

In lateral flow assay, the assay dynamics require an antibody with high affinity. The rationale being that in ELISA a long incubation period (plus 30 minutes) is given for binding of the antibody with homologous antigen. In lateral flow, as the antibody traverses the antigen test line. the binding opportunity is seconds.

Biotinylation of Ab with test line depletion (competitive / inhibition assay) will only work if biotinylated Antibody (Ab) is retained in the MTIST well by binding to Myco spore. The Myco spore is too large to travel through the Lateral flow membrane. If the biotinylated Ab recognises and binds to Myco soluble antigen the biotinylated Ab will get picked up at the test line regardless of whether epitope is bound to Myco soluble antigen or not. Streptavidin is only selective for biotin – it will not discriminate Myco bound biotinylated antibody verus unbound biotinylated antibody

Wet testing of biotinylated Fab gold conjugates

Method

As the Fab was visibly unable to bind to the homologous antigen (*Mycosphaerella*) at the test line steps were made to assess biotinylation of the BSA Fab gold conjugate and capture at the test line using a Polystreptavidin system. Antibody CP137 was diluted in PBS at 1:10, 1:100, 1:1000.

OD10 gold was diluted to OD5 and OD2 in dH2O. Approximately 2ul of each CP137 dilution and 2ul gold dilution were added to 20ul PBST BSA in well of 96 well plate. The strip was run for 5 mins before being removed from the well and photographed.

Results

The results showed good line intensity using 1:10 dilution of conjugate with all 3 test lines clearly visible at OD2. Using 1:100 dilution of CP 137, line intensities diminish considerable across the 3 lines (Figure 13) . At OD2 the majority of the conjugate was captured on the 1st test line leaving the 2nd and 3rd lines weak. Line intensities are very weak with the use of 1:1000 of CP 137 even with the use of OD10 gold Fab. Gold conjugates OD5 and OD10 at 1:10 through to 1:100 were incubated with MTIST trapped *Mycosphaerella* spores up to 5000 spores per microtitre well. No reduction was observed at the test line when probed using Lateral Flow Assay.

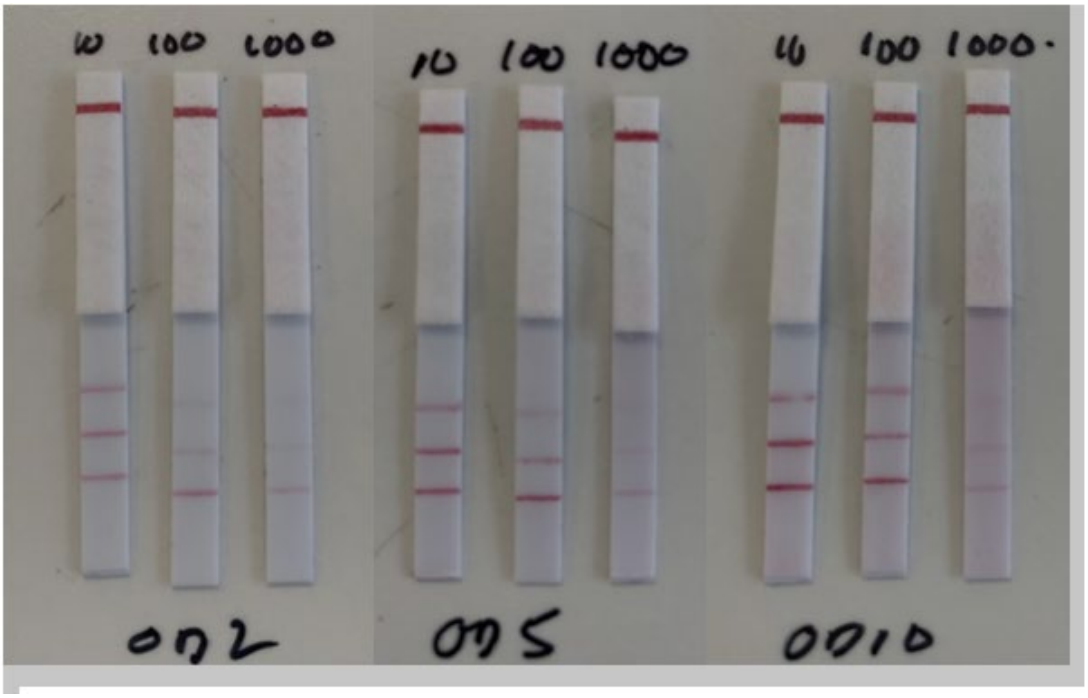

Figure 13 Polystreptavidin test line capture of Biotinylated Gold conjugated Fab

Conclusion

Results show the Fab antibody is caught at the test line. No depletion of test line was observed when the biotinylated Fab was pre-incubated with Myco spores. If Myco ascospore soluble antigen could be prepared and standardized to a concentrate form it would be useful to assess the Fab in a double antibody sandwich format for free soluble antigen. However, ascospore availability was limited to MTIST trapped ascospores. Pairings for use in lateral flow production could include Mab IgM test line capture with Fab conjugated gold. Fab test line and Fab

conjugated gold pairings. This format would rely on repeat epitopes present on the soluble antigen.

Milestone

3.2. Complete the development of a dry lateral flow assay with stability.

A lateral flow could not be developed for measurement of Myco spores using the cell line supplied. However, assessment of AHDB monoclonals identified an IgM/F(ab) Myco antibody which bound to antigen applied at the test line. Five hundred dipstick lateral flow assays were prepared as described in 4.1.

4. Provide commercial Lateral Flow tests for the industry and fully commercialise of test production.

4.1. Establish a supply of commercial *Mycosphaerella* tests

Preparation of Myco lateral flows in dry format

Methods

The following equipment and materials were employed for preparation of 500 dry lateral flows (dip stick assay) for measurement of *Mycosphaerella melonis* ascospores by competitive lateral flow assay.

Material	**Supplier**
Antigen	AW
80mm Backing card	Lohmann,
Sink Pad	Ahlstrom
Sample pad	Whatman
Top laminate (red)	Lohmann
Bottom laminate (clear)	Kenoshatape
Conjugate pad 27mm	Millipore
NC 40mm	MDI
PBS	Sigma
Anti-Mouse	Lampire
50% sucrose PBS	Fisher

Table 10 Preparations and other materials used for LFD production

Preparation of test material

Antigen centrifuged for 5mins at 13,000rpm. Supernatant removed and pellet discarded. Note: No addition of sucrose

Preparation of control line material

Dilute anti-mouse to 1mg/ml

Manufacture

Test line was dispensed at 15mm, 0.1ul/ml, and the membrane dried at 37^0C, 10mm/sec. Following drying, test line antigen was dispensed again and dried. Test line material was dispensed for a 3rd time. The control line was dispensed at 0.1ul/mm (20mm). The manufactured membrane was dried again and stored with desiccant in foil pouches. Laminating of Nitrocellulose test and control lines was as previously reported above.

Conclusions

It was possible through the testing procedure to produce a supply of lateral flow devices which were stable and could be used to detect Myco spores in air samples.

General Conclusions

The work carried out within CP 137a has shown that the Myco pathogen and powdery mildew become prevalent with the second cropping period for cucumbers in the UK. It was confirmed that powdery mildew could be detected immediately after planting in the second crop demonstrating that the pathogen could already present on transplanted seedlings. No powdery mildew was detected on the first cucumber crop making it less likely that the spores from the 1st crop infected the second. It is very likely that Myco infection is also present on the cucumber crop at planting and may originate from seed contamination as transplanted crops are very young.

Isolate variation has been demonstrated for the first time in the response of Myco to different fungicidal active ingredients. The effectiveness of difenoconazole in reducing *Mycosphaerella melonis* growth *in vitro* has also been demonstrated. Difenoconazole is also very effective in controlling *Mycosphaerella brassicicola* on vegetable brassicas. It's unclear if this chemical could get the necessary approval for application on cucumbers. There was little isolate variation in the response to Talius (proquinazid) however both Sercadis (fluxapyroxad) and

Filan (boscalid) showed a high degree of isolate response to these chemicals. It's possible that these chemicals could be used very effectively in some on some cucumber production areas. It was demonstrated that there was no major variation between isolates of those collected in their genotype. There is association between *Mycosphaerella melonis* and isolates of *Phoma.* Further screening of isolates could using primers which recognize *Phoma* could be carried out to check this potential variation..

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