Project title:	Cucumber – Improving Control of Gummy Stem Blight caused by *Mycosphaerella melonis*
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Project leader:	Dr G M McPherson
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Date project completed (or expected completion date):	31 January 2014

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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CONTENTS

GROWER SUMMARY

Headlines

- Methodology to provide growers with an indication of high *M. melonis* risk periods has been developed.

- Several disinfectants have been shown to have good activity at killing spores and mycelium of *M. melonis* in a range of different tests.

- Effective disinfection in between crops can dramatically reduce spore numbers.

- A number of experimental novel fungicide products have been shown to provide excellent control of *M. melonis* in glasshouse trials.

Background

Black stem rot, gummy stem blight or 'Myco' as growers prefer to call it, is caused by the ascomycete fungus *Mycosphaerella melonis* (syn. *Didymella bryoniae*). It is an economically damaging pathogen of cucumber and other cucurbits. Infection of flowers, developing fruit and shoots/stems can occur from air-borne spores. Such infections may become visible in the crop but may, probably under specific environmental conditions, remain latent (hidden) only developing visually once the fruit has been marketed. These 'hidden' infections of fruit, which can sometimes be identified by a tapering to the tip of the fruit, can lead to rejection and reduced retailer and consumer confidence in the product (Figure 1). Effective control of the disease is difficult in intensive production systems and likely to be made worse by recent changes to EU pesticide legislation which have effectively prohibited use of some of the more effective approved fungicides, e.g. triflumizole (Rocket).

Figure 1. Internal *M. melonis* infection in fruit.

An extensive literature review carried out in Phase 1 of this study helped to identify various areas for work. The expected deliverables in Phase 2 were:

- To validate the developed immunoassay system in a semi-commercial crop.

- To carry out *in vitro* screening of experimental products for disease control.

- To further test short-listed products from above under semi-commercial conditions.

- To investigate the efficacy of disinfectants against *Mycosphaerella* to limit secondary spread of infection.

- To investigate the potential for systemic infection under UK conditions.

- To devise an integrated strategy for *Mycosphaerella* control and validate its use in a commercial cropping situation.

Summary

Immunoassay development

Work to develop a sensitive monoclonal antibody (MAb) to *M. melonis* which was started in Phase 1 of this project has progressed well. Two MAbs were identified and one was used to develop an assay for rapid quantification of *M. melonis* spores collected in traps. The assay was tested in a glasshouse crop for reactivity using enzyme-linked immunosorbant assay (ELISA) and Immunofluorescence (IF). Results from spore trapping in a commercial cucumber crop in Yorkshire and a semi-commercial crop at STC during 2011 and 2012 that spore release was significantly greater between 17.30 and 03.00 hrs than at other times during the day/night. This coincides with optimum conditions for infection in the crop when the vents are shut and RH levels are likely to be higher. Sampling in the systemic infection trial at STC during 2012 provided some additional interesting data on the diurnal periodicity of *M. melonis* spore release, which showed that peak spore release occurred between 16.00 and 07.00 hrs. These data are consistent with previously published data.

Initial data on spore release and disease incidence studies from the air-monitoring would appear to indicate that an ascosporic aerosol concentration in excess of 2000 spores/m^3 of air may be required for infection and subsequent disease development.

Glasshouse testing of low risk experimental fungicide and bio-pesticide products

Large scale *in vitro* laboratory screening of a range of novel pesticide and bio-pesticides was carried out in Phase 1 of this project, with promising candidates for control being taken forward into small scale young plant studies in 2011 (Annual Report of HDC Project PE 001a, 2012). Ultimately short listed products were taken forward into a larger, replicated glasshouse study carried out during May - September 2012 at STC. A total of 12 treatments, including a water control, a standard fungicide programme, 8 experimental fungicides and 2 bio-pesticide programmes, were used. Bio-pesticides were applied weekly with 9 applications in total, whilst conventional fungicides were applied fortnightly with a total of 4 applications. Guard plants in the crop were inoculated with *Mycosphaerella* following the 1st conventional fungicide application (and after 2 bio-pesticide applications). The guard plants were inoculated a second time, and infected detached fruit was introduced into the cropping area to ensure high disease pressure via ascospores release. The crop was assessed for the incidence and severity of *Mycosphaerella* lesions on three occasions (monthly) following the 1st conventional fungicide application, with the final assessment being carried out one month after the final application (Figure 2).
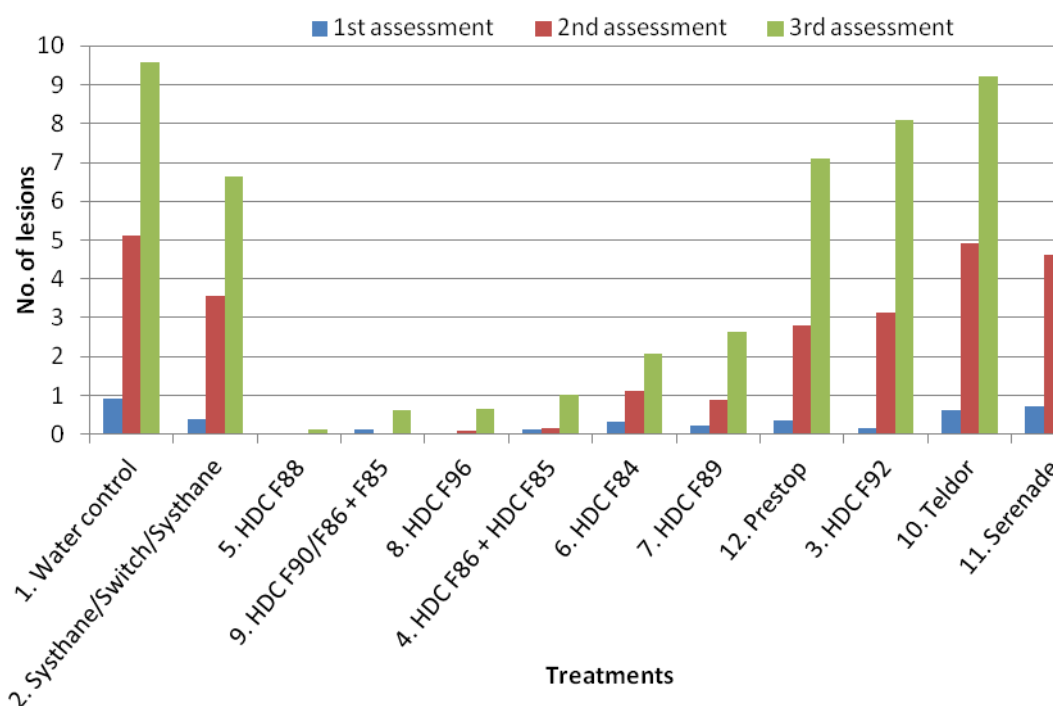

Figure 2. A comparison of the mean number of stem lesions of *M. melonis* per plant at each assessment date.

The collected data shown in Figure 2 indicate that during the first assessment, only very low disease levels were present but, as the season progressed and inoculum levels increased, infection levels rose and excellent treatment differences developed. Relative to the water control, none of the <u>approved</u> products or either of the bio-pesticide products tested prevented *Mycosphaerella* development in this study. Though it is important to note that all these products don't necessarily have a label approval for this target. However, several of the experimental products under investigation showed good efficacy against *Mycosphaerella* e.g. HDC F85 + F86, F88, F89, F90 and F96. A slight crop safety issue was observed following the first application of F88 and F89 when applied to younger plants.

Limiting secondary spread of infection using disinfectants

A series of experiments was undertaken to identify disinfectants with good activity against *M. melonis*. Six disinfectant products containing active ingredients from different chemical classes were tested for activity against conidia and mycelium of the fungus. Products were tested at their full recommended rate and at half-rate after exposure for 5 mins and 30 mins. Jet 5 (hydrogen peroxide/peracetic acid) and Fam 30 (iodophor) were most effective. These products, together with bleach (sodium hypochlorite) and Unifect G (glutaraldehyde + Quaternary Ammonium Compound, QAC) were fully effective after just 5 mins and at half their recommended rates. Menno Florades (benzoic acid) was effective after 5 mins at full rate and after 30 mins at half rate; Vitafect (QAC + biquanidine salt) was effective at full rate but ineffective at half rate even after 30 mins. The most effective products against mycelium in filter paper discs were Jet 5, bleach, Unifect G and Vitafect.

An experiment was designed and undertaken to examine the influence of different surfaces on the activity of disinfectants against *M. melonis*. Overall, perhaps not surprisingly, it was more difficult to disinfect concrete than aluminium, glass or plastic. Jet 5, bleach and Unifect G used at their recommended rates were fully effective on all four surfaces. However, Fam 30 on concrete, Menno Florades on aluminium and concrete, and Vitafect on glass all showed reduced activity.

An experiment was done to determine how effective various disinfectant soak treatments were at reducing disease transmission of *M. melonis* on knives contaminated with the fungus by cutting through infected cucumber leaves and stems. Disease transmission was relatively low. Soaking contaminated knives in water, Jet 5, Menno Florades, bleach or Vitafect for 1 hour reduced the development of gummy stem blight in cucumber fruit slices compared with transmission from untreated knives. Results of all the disinfection tests described above are summarised in

Table **1**.

Table 1. Summary of disinfectant activity against *M. melonis* in various tests - 2011

Disinfectant	Rate used	Growth of *M. melonis* recorded after treatment[a] of						
		Spores* in water	Mycelium on filter paper in water	Spores*/mycelium dried on:				Dirty knife [b]
				Alu	Con	Gla	Pla	
Water (control)	N/A	+	+	+	+	+	+	(+)
Fam 30	1:125	-	(+)	-	+	-	-	NT
Jet 5	1:125	-	-	-	-	-	-	(+)
Menno Florades	10 ml/L	-	+	(+)	+	-	-	-
Sodium hypochlorite (10-14%)	1 in 10	(+)	-	-	-	-	-	(+)
Unifect G	4%	-	-	-	-	-	-	NT
Vitafect	1%	-	-	-	-	(+)	-	(+)

[a] Results shown after exposure to disinfectant for 5 mins (spores or filter paper in water) or 30 mins (all other tests).

[b] Disease transmission test.

N/A – not applicable; NT – not tested.

-    no growth; (+) occasional growth; + growth common.

Alu – aluminium; Con – concrete; Gla – glass; Pla – plastic

* The spore type evaluated was not differentiated though considered to comprise largely of conidia rather than ascospores

Two experiments were carried out to compare different treatments for cleansing hands contaminated with *M. melonis* following handling of cucumber fruit affected by *M. melonis*, and through contamination of hands with a paste of the fungus in cucumber sap. A finger from a washed hand was placed on a culture plate to check for pathogen viability. Washing hands in soap and water, with an alcohol gel, or with alcohol foam, all greatly reduced transmission of *M. melonis* from hands. Soap and water alone was less effective at reducing transmission of *M. melonis* than soap and water followed by alcohol gel or foam, or the alcohol foam or gel used directly on contaminated hands. Rinsing hands in water alone gave no reduction in transmission of *M. melonis*.

Systemic infection potential

A glasshouse trial to investigate the potential for systemic shoot infection by *Mycosphaerella* was undertaken during 2012. Tagged plants were artificially inoculated in

different sites; leaf petioles (agar plug), cut fruit stubs (agar plug), main stem wound of stopped plant at the wire (agar plugs and spore suspension), flowers (spore suspension) and shoot tips of laterals (spore suspension) using either a *Mycosphaerella* spore suspension or agar plugs from an actively growing culture. A spore suspension of the pathogen was also drenched into the rock-wool block. Symptom development was compared with that on uninoculated control plants. The incidence and severity of any lesions that subsequently developed was recorded during the growing season.

All artificial inoculation techniques on the plants led to the development of more *Mycosphaerella* infections than would have occurred naturally. Inoculation of the leaf petioles, cut fruit stubs and main stem wound at the wire resulted in the development of aggressive stem lesions. Inoculation of the flowers resulted in the development of infected fruit and inoculating the shoot tips of laterals caused the tip to 'burn out' and the lateral shoot to become weaker (Figure 3). The *Mycosphaerella* spore suspension drenched into the rock-wool blocks did not result in a greater infection level than occurred naturally on the uninoculated control plants. This suggests that infection cannot occur via root uptake, though applying the drench at earlier growth stages may give different results.

The majority of weak laterals that developed were recorded on plants where the shoot tips were sprayed with a spore suspension although it is also worth noting that weak laterals were also recorded, albeit to a much lesser extent, on plants inoculated using other methods and also on the uninoculated plants.

Figure 3. Good example of 'burnt out' tip of a weak lateral.

The laterals that appeared weak with 'burnt out' tips were analysed for the presence of *Mycosphaerella* in the internal stem tissues, and this was compared with *Mycosphaerella* presence in uninoculated symptomless shoots. Whilst *Mycosphaerella* was successfully isolated from the nodes of both inoculated and uninoculated laterals its rate of recovery

from internodes of inoculated laterals was much greater than from internodes of uninoculated laterals.

Whilst it is difficult to draw firm conclusions from this study it would appear from these artificial inoculation studies that the cucumber shoots can become infected with *Mycosphaerella* internally leading to the development of weak unproductive shoots. Such infection would appear to occur as a direct result of spores infecting the young shoot tips of the same laterals. The presence of the pathogen internally in uninoculated plants could have occurred as a direct result of ascospore release in the glasshouse as the epidemic developed following artificial inoculation.

Integrated strategy for *Mycosphaerella* control (Immuno-assay, disinfectants, fungicides)

The final component of the work which seeks to develop an integrated disease control strategy using all the new knowledge regarding spore release and infection risk, use of disinfectants and deployment of effective fungicides in commercial crop situations will be carried out in the summer of 2013.

Financial Benefits

The results from the disinfectant study carried out during 2011 will have immediate benefits for growers both during the growing season and during the clean-down between crops. Effective use of disinfectants should help to reduce disease spread and the survival of inoculum between crops. Several experimental fungicides have been shown to provide effective control of *M. melonis* in fully replicated glasshouse studies, these products are not yet approved for use in cucumbers and therefore cannot yet be used commercially. However, feedback from the various manufacturers remains encouraging and it is hoped that one or more products will be available, in 2014, subject of course to the usual regulatory process either by on-label or via a minor use approval (EAMU).

It is also worth noting that some of the experimental products which showed good activity against *M. melonis* also showed activity against powdery mildew and this would result in even greater financial benefits for the industry, as it would potentially allow effective resistance management strategies to be deployed thus safeguarding products for the longer term.

Action Points

- Consider implementing the use of the disinfectants identified as having good efficacy during crop production and for the clean down between crops.

- Ensure the use of good quality seed from reputable suppliers, and be aware of the potential for seed-borne risk on new cultivars.

- Be aware of the relatively low activity of the approved products against *Mycosphaerella* observed during this study and recognise that *Mycosphaerella* control will continue to present a challenge with the current armoury of 'powdery mildew' fungicides.

- The use of tolerant cultivars integrated with effective hygiene and disinfecting will continue to be very important until such time that alternative effective products are available for *Mycosphaerella* control.

SCIENCE SECTION

Introduction

Gummy stem blight caused by *Mycosphaerella melonis* (*M. melonis*) has been a persistent leaf, stem & fruit disease in glasshouse cucumber for many years (Figure 4). It has been generally suppressed, rather than controlled, over the years using a combination of rigorous hygiene precautions (to remove debris that might otherwise allow the pathogen to carry-over from crop to crop in the glasshouse), environmental manipulation (to avoid conditions conducive to infection), use of fungicides (to prevent infection and spread of the pathogen) and more recently through the use of better cultivars (to reduce the rate of disease progression in the host crop). However, more recently, a number of factors have impacted on the disease and it is becoming more prevalent and damaging economically with fewer opportunities for effective control, once established in the crop.

This is of considerable concern for growers due to the potential economic damage this pathogen can cause either through direct loss of plants, reduced plant vigour and/or fruit infection. Increased energy costs are a significant factor leading to increased infection as growers become increasingly constrained over the ability to use pipe heat early in the morning to dry the foliage and therefore avoid conditions conducive to infection. Similarly, the loss of key active substances as a result of the EU pesticide review programme has meant that growers have fewer useful products with good activity against the pathogen to prevent infection e.g. triflumizole (Rocket). This is further influenced by the increased shift in consumer (retailer) perception regarding pesticide residues. An indirect impact of all this is the increased use of cultivars with tolerance to powdery mildew (where most fungicides are usually used for

Figure 4. *M. melonis* stem lesion (picture courtesy of D. Hargreaves).

10

control). This means that growers are applying fewer fungicide sprays which otherwise would have provided incidental control, or at least suppression, of *Mycosphaerella* infections. There is also some evidence to suggest that such mildew tolerant cultivars are actually more susceptible to *Mycosphaerella*.

No recent studies have been undertaken in the UK to determine the sensitivity of existing and/or new fungicides and bio-control products against *Mycosphaerella* and growers have to rely on an ever diminishing armoury of products. Unless we can find alternative approaches to the control of such endemic pathogens we could potentially expect a continued increase in disease, potentially reaching epidemic proportions.

This project aims to seek alternative control strategies. This includes the evaluation of disinfectants, novel fungicides & alternative bio-control products and the use of novel immuno-assay or serological techniques to predict disease risk by monitoring the pathogen spore population in the glasshouse in order to take action before infection is allowed to occur; thereby improving application timing to prevent economic loss due to the disease.

Materials and methods

Development of Monoclonal Antibody Cell Lines to ascosporic inoculum of *M. melonis*.

From six hybridoma fusions, nine cell lines were identified with reactivity to *M. melonis* from more than 1100 hybridoma cell lines tested. However of these, only two tested positive to the ascosporic stage of the pathogens life cycle and were cloned twice to monoclonal stage (MAb). Reactivity studies to determine the suitability of these MAbs within a glasshouse setting, for the selective identification of airborne *M. melonis* ascospores, was assessed by enzyme-linked immunosorbant assay (ELISA) and Immunofluorescence (IF). A cell line (coded 2B11 D1 D2) was selected to develop an assay format for rapid quantification of trapped airborne inoculum of *M. melonis*. The cell line was isotyped as IgM class. The cell line did not react to the conidial stage of *M. melonis* but did so to ascospore and the hyphal tip (Figure 5). It was determined from this that collected aerosols would require inhibition of spore germination to prevent over estimation of the trapped target inoculum by immunoassay.
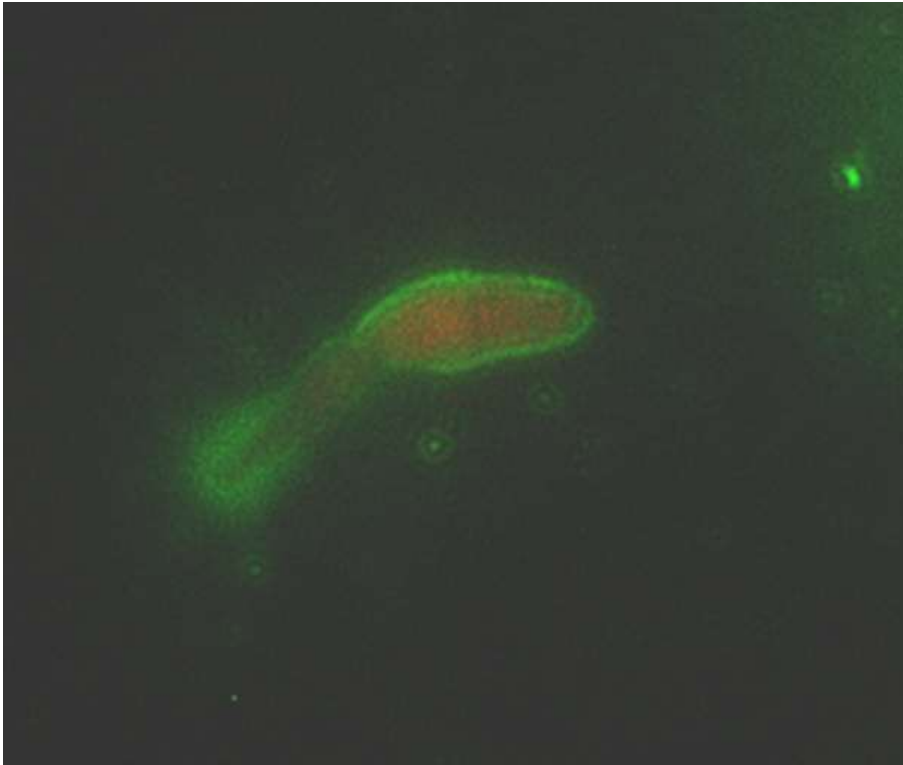
Figure 5. Reactivity of cell line 2B11 D1D2 to ascosporic disease inoculum of *M. melonis* as viewed by Immunofluorescence

Evaluation of immunoassay air sampling systems to monitor airborne disease inoculum of *M. melonis* in a glasshouse cucumber cropping system

Commercial glasshouse cucumber crop, Yorkshire (2011). Over a four month period (August-December), ascospores of *M. melonis* were monitored in the air of a commercial glasshouse cucumber cropping system by two air sampler types. A seven day volumetric air sampler was placed at ground level and adjacent to a microtiter immunoassay air sampler (MTIST) (Figure 6). The volumetric sampler operated continuously at an air flow rate of 10L minute^{-1}. A Melinex tape fixed to a rotating drum and positioned inside the volumetric spore trap operated for multiple seven day periods where air particulates were impacted directly onto the tape. Every 7 days the Melinex tape was removed, sent to the National Pollen and Aerobiology Research Unit, Worcester (NAPRU) and sectioned into 24 hr periods. Under bright field microscopy and at a magnification of x400, each of the weekly tape sections were examined for the presence of ascospores of *M. melonis*. It should be noted that by light microscopy it may not be possible to differentiate between ascospores within the *Didymella* genus or morphologically similar spore types.

12

Figure 6. Air sampling equipment

Two indoor personal MTIST air samplers were positioned within the crop at ground and canopy height (Figure 6). The air samplers operated continuously and at an air flow rate of 57 L air minute^{-1}. Air particulates (to include fungal spores) were collected on the base of 4 x 8 well microtiter well strips. The microtiter strips were pre-coated with sodium azide to inhibit germination of trapped biological material. At weekly intervals the microtiter strips were changed and the glasshouse exposed strips stored at -20°C. At the end of the four month sampling period the strips were processed by plate trapped antigen ELISA (PTA ELISA) for quantification of collected disease aerosols of *M. melonis.* The MAb 2B11D1D2 was included within the assay format to label and identify inoculum of *M. melonis.*

Air sampling in an artificially Inoculated Cucumber Crop (STC, 2012)

Additional air sampling was carried out in the systemic infection trial glasshouse at STC. The crop was inoculated in a number of ways on the 8th, 19th and 29th June 2012 (see section below for full details). A volumetric seven day air sampler, positioned at the base of the crop, collected daily aerosols during the study period (June to September 2012).

MTIST air samplers were also operated within the crop for the duration of the experiment positioned as in previous crops at the base and at the height of the crop canopy. Quantification of the MTIST collected *M. melonis* ascospores in the weekly crop aerosols was determined by immunoassay (PTA ELISA) using MAb 2B11. The absorbance values generated were compared to the weekly microscopic counts.

Testing of low-risk experimental fungicides and bio-control products

Extensive *in-vitro* and small-scale *in-planta* testing carried out in year 1 of this project provided us with a short list of products with good potential for *M. melonis* control. A large, fully-replicated study was undertaken in a glasshouse at STC between May and September 2012.

A cucumber crop cv. Shakira was established in a multi-factorial glasshouse unit (MFU) on the 14 May 2012. The crop was grown in double rows to a 2.2m (8ft) wire, where it was stopped. Three lateral shoots were allowed to develop. The plots treated with the biological control products (Serenade ASO and Prestop) were positioned to one side of the glasshouse to minimise potential interactions with conventional fungicides e.g. drift or vapour effect, which might otherwise compound the results (see trial plan – Appendix 1).

Treatments

Table 2. List of treatments employed in the fungicide/bio-pesticide trial 2012

Treatment No.	Product name/HDC code	Active ingredient	Application rate
1	Water control	-	-
2 (standard programme)*	Systhane/ Switch/ Systhane	myclobutanil/ cyprodinil + fludioxonil/ myclobutanil	0.375L/Ha 200ml/100L
3	F92	-	-
4	F85+F86	-	-
5	F88	-	-
6	F84	-	-
7	F89	-	-
8	F96	-	-
9	F90/F85+F86	-	-
10	Teldor^	fenhexamid	1g/L
11	Serenade ASO^	*Bacillus subtilis*	10L/Ha
12	Prestop^	*Gliocladium catenulatum*	500g/100L reduced to 375g/100L

* Agreed following consultation with Mr D Hargreaves:
- Apply Systhane as 1st foliar spray post-planting
- Apply Switch as a stem base spray (basal 60-100cm) after 5-7 days
- Repeat foliar sprays with Systhane (twice)

^ Approved products tested do not necessarily claim activity against *Mycosphaerella*, so in situations of poor control this in no way reflects negatively on these products.

Crop Diary

14.5.12	Cucumber cv. Shakira planted
24.5.12	1st biological product (BP) application
31.5.12	2nd BP and 1st fungicide (F) application
1.6.12	Guard plants at either end of each plot inoculated with *Mycosphaerella*
7.6.12	3rd BP application and infected detached fruit introduced to boost inoculum
11.6.12	Guard plants inoculated with *Mycosphaerella*
14.6.12	4th BP and 2nd F application
19.6.12	Guard plants inoculated with *Mycosphaerella*
21.6.12	5th BP application
26.6.12	Guard plants inoculated with *Mycosphaerella*
28.6.12	6th BP and 3rd F application
3.7.12	1st disease assessment
5.7.12	7th BP application
12.7.12	8th BP and 4th (final) F application
19.7.12	9th (final) BP application
26.7.12	2nd disease assessment
14.8.12	3rd disease assessment

During each disease assessment the 7 central plants/plot were assessed for *Mycosphaerella* and additional data were collected on the incidence of *Botrytis cinerea* (grey mould) in the crop. A number of different parameters were assessed including number and type of node infections, number and type of wound infections, number of infected fruit, and number of weak lateral shoots.

Limiting secondary spread of infection using disinfectants

This component of the investigation has been completed and was reported in full in the 2012 annual report.

Investigating the potential for systemic infection

Previous observations and ad hoc investigations (McPherson, G.M., personal communication) on the production of weak, spindly lateral shoots (Figure 3) have suggested that they may be associated with an internal Mycosphaerella infection and this has led to a hypothesis of a potential systemic infection being present in plants, which may or may not, depending on plant stress and environmental conditions, either develop into fully symptomatic *Mycosphaerella* infections or else remain as weak unproductive shoots limiting

Figure 7. Systemic infection trial *in situ*. Spore trapping equipment in position**.**

the full crop potential in terms of yield. This study provided an opportunity to further investigate this hypothesis through specific inoculations of plants with *M. melonis* using a variety of different techniques and monitoring disease development visually and by taking tissue samples for laboratory testing (culturing) for the presence of symptomless infections.

Cucumbers of cv. Shakira were raised and planted in rock-wool slabs in a run-to-waste system in glasshouse F19 at STC in May 2012. Burkard and MTIST spore trapping equipment was also located in the same crop to gather further data for the immunoassay development work being carried out by project colleagues at Worcester University (Figure 7).

Inoculation with a virulent strain of *M. melonis* was carried out using a variety of techniques at different stages of crop development and these served as different 'treatments' in this study. Agar plug inoculations were made using a 1 cm diameter disc of actively growing mycelium on Potato Dextrose Agar (PDA). 2 ml conidial suspension containing approximately 4 x 10^5 viable conidia, verified by colony counts on PDA plates, was applied to each spray inoculation site. 10 ml conidial suspension containing approximately 2 x 10^6 viable conidia, verified by colony counts on PDA plates, was applied to each drench inoculation site.

1. Agar plug inoculation of leaf petioles (1 petiole/plant) 2-4 weeks post-planting and again 6 weeks post-planting.
2. Conidial suspension sprayed on lateral shoots 2-4 weeks post-planting.
3. Conidial suspension drenched onto the rockwool block 2-4 weeks post-planting.
4. Not inoculated
5. Not inoculated.
6. Conidial suspension sprayed onto open flowers (3 flowers/plant) 2-4 weeks post-planting.

7. Agar plug inoculation to the main stem when the plant was stopped at the wire 6 weeks post-planting.

8. Conidial suspension sprayed onto the main stem wound when the plant is stopped at the wire 4 weeks post-planting.

9. Agar plug inoculation of cut fruit stubs during harvest (3-6 stubs/plant) 6 weeks post-planting.

10. Conidial suspension sprayed onto the shoot tips of laterals soon after they have developed following the plant being stopped 4 weeks post-planting.

Ten randomly positioned plants were inoculated per treatment described above (see plan, Appendix 2). All inoculated plants and inoculation sites were tagged using coloured ribbon so that they could be tracked effectively as the crop developed.

Crop Diary

18.5.12	Crop established
8.6.12	Inoculation with agar plugs (T1)
12.6.12	Spore trapping commenced
19.6.12	Inoculation with conidial spore suspension (2, 3, 6, 8 & 10)
29.6.12	Inoculation with agar plugs (T1, 7 & 9)
2.7.12	1st disease assessment made
18.7.12	2nd disease assessment made
13.8.12	Laterals sampled and stem pieces plated out
16.8.12	3rd disease assessment made
7.9.12	Final disease assessment made
2.10.12	Laterals sampled and stem pieces plated out
9.11.12	Laterals sampled and stem pieces plated out
12.11.12	Crop terminated

The crop was assessed four times for the development of lesions but by the fourth assessment more than 50% of the plants had died as a result of severe *Botrytis* infections so these data have not been included in the analysis as it was difficult to assess many of the parameters on the dead plants.

Weak laterals with 'burnt out' tips and healthy laterals were sampled from the crop. The stems were surface sterilized, cut into sections and sliced transversally into discs. The outer stem tissues were removed aseptically, leaving the internal tissues which were then plated out onto PDA agar. The stem pieces were then monitored for the growth of

characteristic *Mycosphaerella* mycelium. If there was any doubt over the black mycelial growth, it was examined microscopically for confirmatory purposes.

Integrated strategy for *Mycosphaerella* control

Due to unforeseen circumstances with the serological work, the final component of the study to develop an integrated strategy for Mycosphaerella control could not be undertaken during 2012. Therefore an extension to the project has been sought such that this important work can be undertaken during 2013. It is proposed that two commercial sites in East Yorkshire – Essex (Lea Valley) will be chosen and the disinfection, immunoassay spore detection and novel fungicides evaluated in an integrated strategy as a demonstration of the potential benefits of the various work components. To ensure high disease pressure this final study will be undertaken in the third crop in 2013 during August-October.

Results and Discussion

Evaluation of immunoassay air sampling systems to monitor airborne disease inoculum of *M. melonis* in a glasshouse cucumber cropping system

Commercial glasshouse cucumber crop, Yorkshire (2011).

Disease control measures were applied throughout the four month period and little disease was observed within the crop. Results of the two air sampling processes are shown in Figure 8, Figure 9 & Figure 10.

Ascosporic inoculum was first observed in the crop at STC from the 6th July. On the 17th July 100 plants were examined and 72 identified as infected. Of these 14 were observed to occur as a result of natural infection. Lesions derived from earlier inoculation processes were recorded with a mean stem lesion of 14cms on the 17th July and were of sufficient maturity to release airborne ascospores in to the air. A peak in ascospore concentration of 2000 spores per cubic metre was recorded in the crop on the 21st July. Uniform and severe disease development was later observed in the crop. Worked carried out previously under optimal controlled environmental conditions found a correlation between the amount of ringspot disease on exposed plants and trapped ascospore numbers of *Mycosphaerella brassicicola*. For significant disease to occur and uniform across the plants > 2000 spores per cubic metre of air sampled were required. (Kennedy *et al.,* 2000).
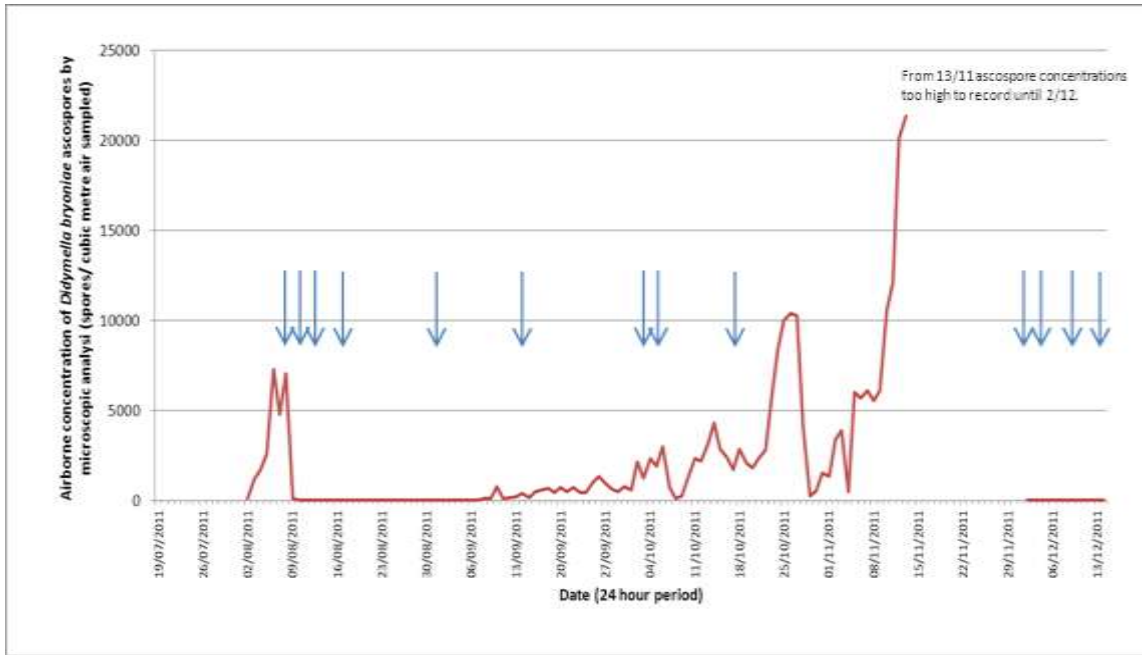
Key Dates

- Crop taken out on 9 th August 2011
- New crop planted 10th August 2011
- Amistar applied 11th August
- Bravo applied 15th August
- Systhane applied 31st August
- Systhane applied 15th September
- Switch / Chalk applied 4th October
- Rocket applied 5th October
- Crop taken out 18th October
- Polythene out on 2nd December
- Pressure wash glasshouse on 5 to 10th December
- Horticide spray treatment 10th to 11th December
- Polythene down on 14th December

Figure 8. Monitored daily aerosols (m^3 air sampled) of *M. melonis* using a volumetric air sampler and identified by bright field microscopy, 2 August - 22 December 2011.
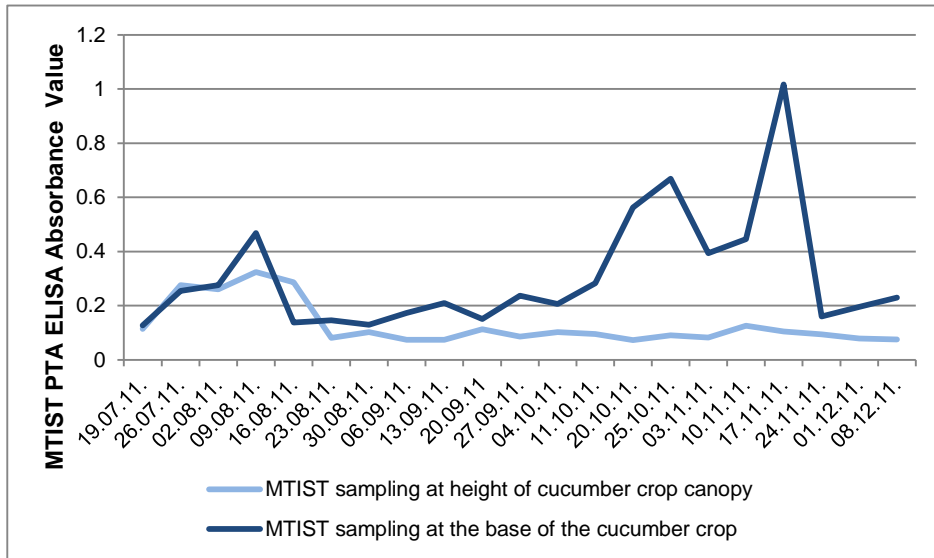
Figure 9. Monitoring MTIST trapped weekly aerosols for *M. melonis* ascospores at canopy height and crop base by PTA ELISA (Immunoassay), 19 July - 8 December 2011.
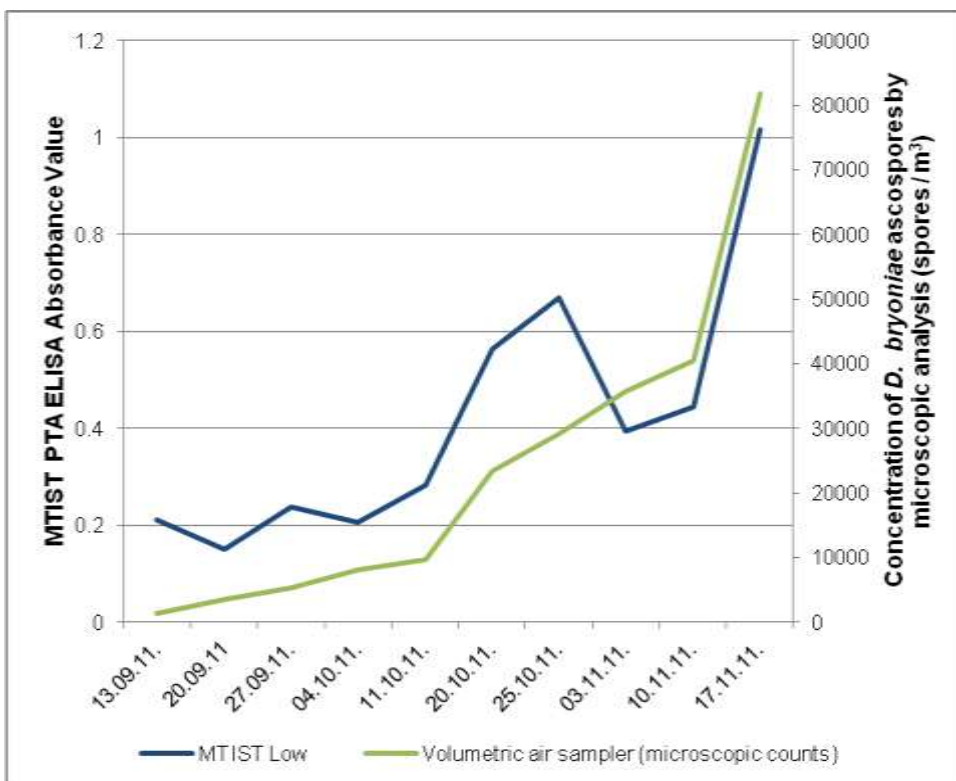

Figure 10. Monitoring weekly aerosols for *M. melonis* ascospores at the base of the crop using a volumetric and MTIST air sampler, 13 September - 17 November 2011.

Air sampling in an artificially Inoculated Cucumber Crop (STC, 2012)

Air samplers were placed in a cucumber crop at STC which was not treated with any fungicides so the natural progression of the disease could be monitored. The assessment of 100 cucumber plants on the 2nd July identified 13 infected plants, the majority of which had occurred as a result of artificial inoculation. The mean length of a stem lesion was 3 cm. A further assessment of 100 plants on 17th July identified 72 infected plants, 14 occurring as a result of natural infection. Between the 2nd and 17th July a significant increase in new lesions was observed on non-inoculated plants.

Ascosporic inoculum of *Mycosphaerella melonis* was first identified in the crop from the 6th July (Figure 11) by bright field microscopic analysis. Figure 12 uses a logarithmic scale to illustrate the disease cycles of the pathogen. It shows there was an early disease cycle from 3rd to 13th July, before the main epidemic became established.

Table **3** shows the daily recorded times of *M. melonis* ascospores in the crop during this initial phase of the disease epidemic. Spore release probably coincides with optimum conditions for infection in the crop when the vents are shut and relative humidity levels are likely to be higher. These data are consistent with data published by Van Steekelenburg (1983).

It should be noted that by light microscopy it is difficult to differentiate between ascospores within the *Mycosphaerella* genus or morphologically similar ascospores. The microscopist noted that from the 16th July onwards significant fungal spore populations were identified (Rusts and Smuts, *Alternaria*, *Pyrenopeziza*, *Leptosphaeria*, *Penicillium*, *Tilletiopsis*, *Cladosporium*, *Erysiphe, Sporobolymyces, Basidiosphores, Botrytis* and other *Didymella* species) in the collected aerosols. A high concentration of ascosporic fungi was recorded which may have led to an overestimation of *M. melonis*.
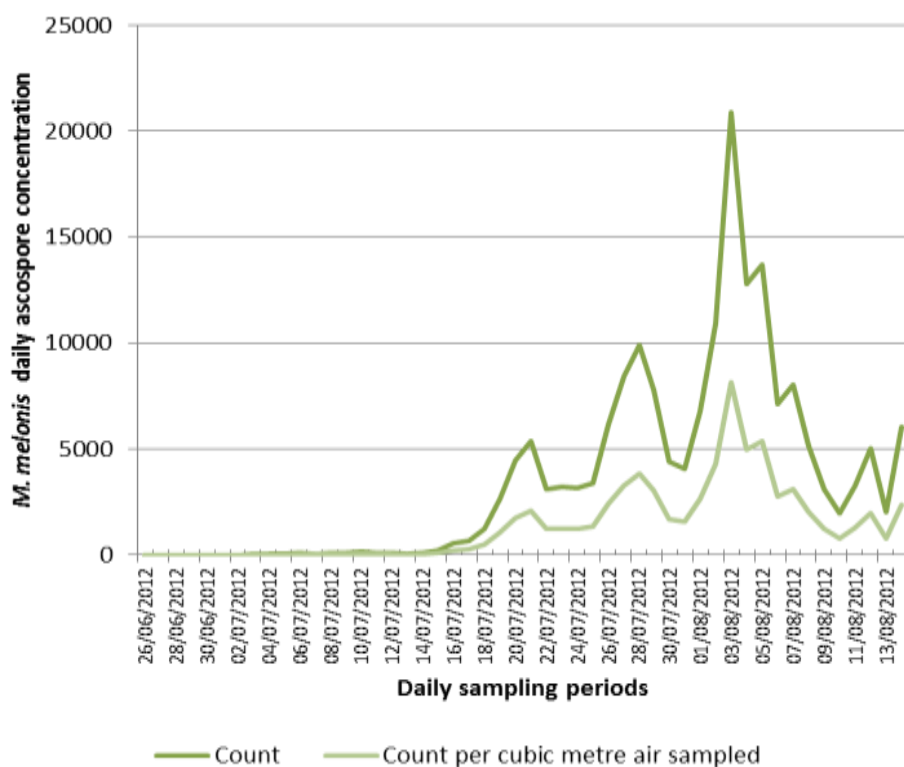

Figure 11. Monitoring daily glasshouse air samples at the base of a cucumber crop for ascosporic inoculum of *M. melonis*, as recorded by bright field microscopy.
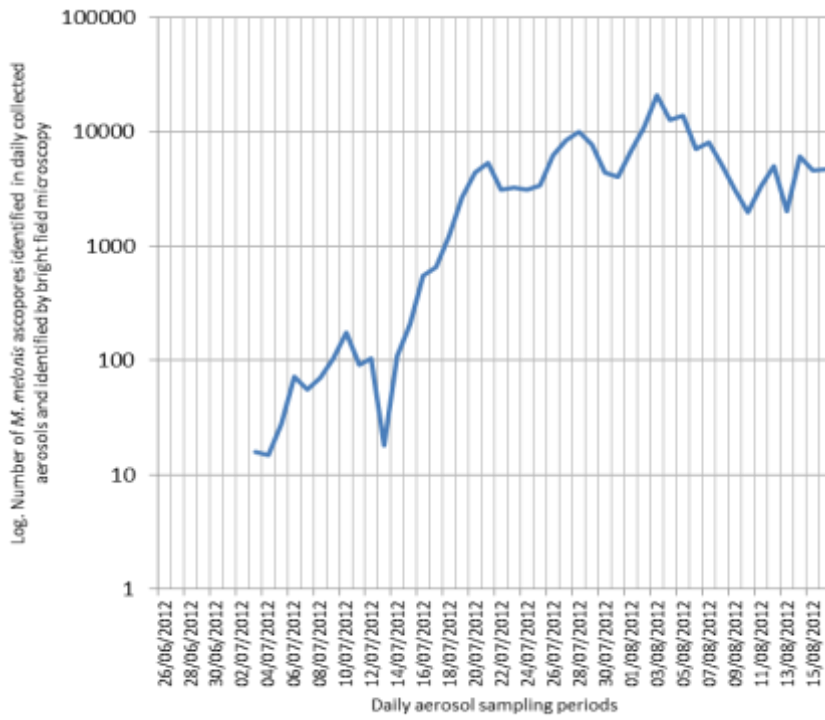
Figure 12. Daily *M. melonis* ascospore counts from glasshouse air samples as recorded by bright field microscopy and represented on a logarithmic scale.

Table 3. Diurnal periodicity of ascospore release

Date	Time *M. melonis* ascospore release detected in the crop
06/07/2012	20.00-22.00
07/07/2012	20.30-22.00
08/07/2012	19.00-24.00
09/07/2012	19.00-24.00
10/07/2012	16.30-03.30
11/07/2012	20.30-24.00
12/07/2012	20.30-23.30
13/07/2012	20.30-23.30
14/07/2012	20.00-07.00
15/07/2012	21.30-09.00
16/07/2012	19.00-01.00
17/07/2012	19.00-01.00
18/07/2012	21.00-01.00
19/07/2012	16.00-04.00
20/07/2012	17.30-04.00
21/07/2012	19.30-07.30
22/07/2012	22.30-09.00
23/07/2012	21.00-09.00

Quantification of the MTIST collected *M. melonis* ascospores in the weekly crop aerosols was determined by immunoassay (PTA ELISA) using MAb 2B11. The absorbance values generated were compared to the weekly microscopic counts (Figure 13). A correlation of 0.9088 was observed between the two MTIST sampling systems (Figure 14). There appeared little difference in vertical concentration of *M. melonis* ascospores within the crop canopy as shown by slightly conflicting results between two spore traps. However, in the STC trial, where artificial inoculation took place at variable heights in the crop, this may have given a more variable positioning of spore release than might occur from naturally occurring infections developing on fruit or stem bases in an uninoculated crop. Both MTIST air samplers recorded an increase in absorbance values after week 2 (26th June onwards), although the crop was inoculated on 19th June with conidia rather than ascospores. Nevertheless each of the three air sampling systems detected significant airborne disease concentration of *M. melonis* after the 16th July (Figure 13). Initial lesions derived from earlier inoculation processes were recorded with a mean stem lesion of 14cms on the 17th July. These lesions were of sufficient maturity to release airborne ascospores inoculum into the

air. Uniform and severe disease distribution within the cucumber crop was recorded thereafter.

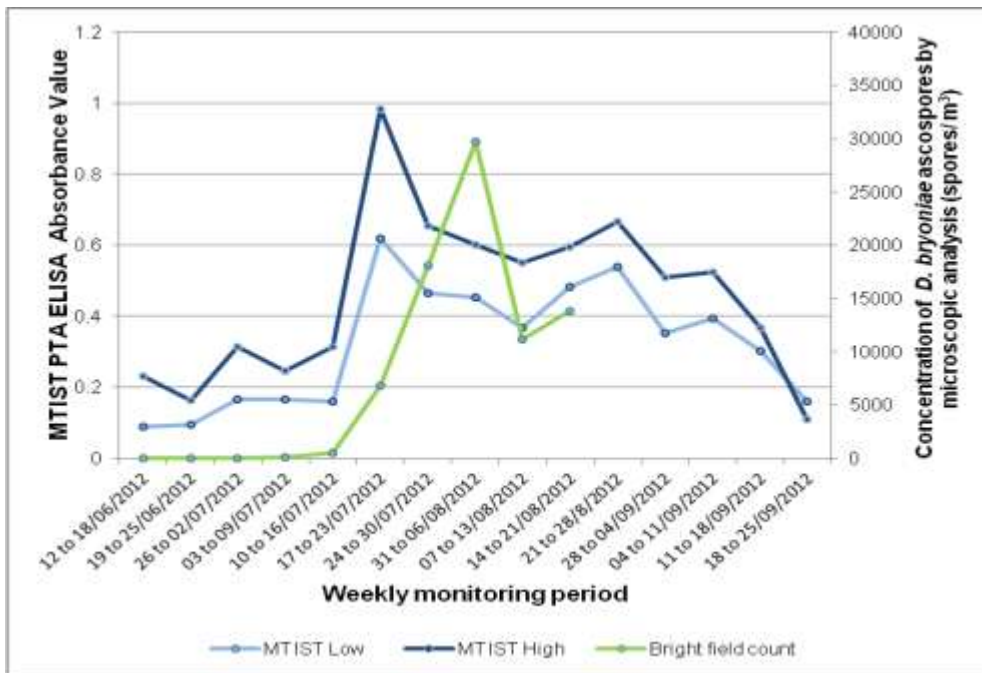**Figure 13.** Comparative sampling of *M. melonis* in collected aerosols of a cucumber crop at STC in 2012: Conventional volumetric 7 day sampler (microscopic counts) and MTIST air samplers (immunological quantitative measurements).
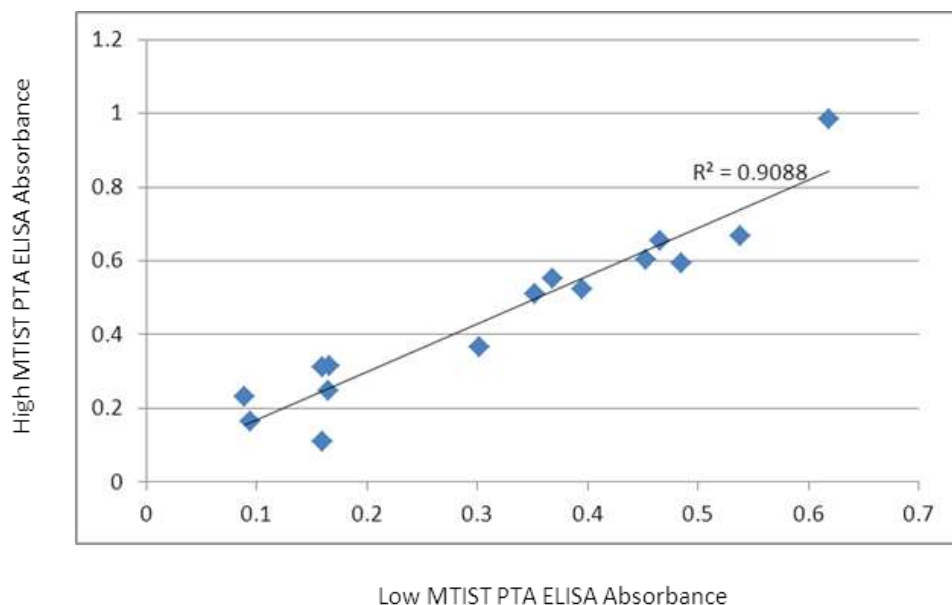

Figure 14. Evaluation of positional measurement of *M. melonis* ascospores in collected MTIST aerosols of an artificially infected cucumber crop over an 8 wk sampling period

Testing of low-risk experimental fungicides and bio-control products

Following initial product application to young plants, two incidences of phytotoxicity were observed. White speckling was observed on the leaves of the plants inoculated with HDC F88 and F89 (Figure 15). As the effect was very minor, it was not considered necessary to dilute the chemical in following applications and no further flecking was observed following later applications with these same products. Following the fourth application of Prestop, severe chlorotic patches were observed on the leaves of the plants (Figure 15). The product rate provided was as a rate per litre of water, rather than a rate per hectare. As the plants were growing the volume of water per plot was increasing at each application, and also therefore the amount of Prestop, so the decision was made to reduce the rate from 500g/100L water to 375g/100L water.

Figure 15. Phytotoxic effects observed on leaves of plants treated with HDC F88 (top left), HDC F89 (top right) and Prestop (bottom row)

As the disease developed following inoculation a number of different infection parameters were assessed. The parameter which showed the most noticeable differences between treatments was the number of *Mycosphaerella* lesions on the main stem compared to the

uninoculated plants. At each assessment these differences were highly significant. The results are summarised in Table 4 and graphically represented in

Figure **16**.

Table 4. Mean no. of stem lesions per plant caused by *Mycosphaerella melonis* at each assessment.

Treatment	Mean No. of main stem lesions per plant					
	1st assessment		2nd assessment		3rd assessment	
1. Water control	0.9	a	5.1	a	9.6	a
2. Systhane/Switch/Systhane	0.4	abc	3.6	abc	6.6	a
3. HDC F92	0.1	bc	3.1	bc	8.1	a
4. HDC F86 + HDC F85 (SDHI)	0.1	bc	0.2	e	1.0	cd
5. HDC F88 (SDHI)	0.0	c	0.0	e	0.1	d
6. HDC F84 (SDHI)	0.3	abc	1.1	d	2.1	bc
7. HDC F89 (SDHI)	0.2	bc	0.9	d	2.6	b
8. HDC F96 (SDHI)	0.0	c	0.1	e	0.6	cd
9. HDC F90/F86 + F85 (SDHIs)	0.1	bc	0.0	e	0.6	cd
10. Teldor	0.6	ab	4.9	ab	9.2	a
11. Serenade	0.7	ab	4.6	ab	9.5	a
12. Prestop	0.4	abc	2.8	c	7.1	a
F probability	0.0001		0.0001		0.0001	

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)
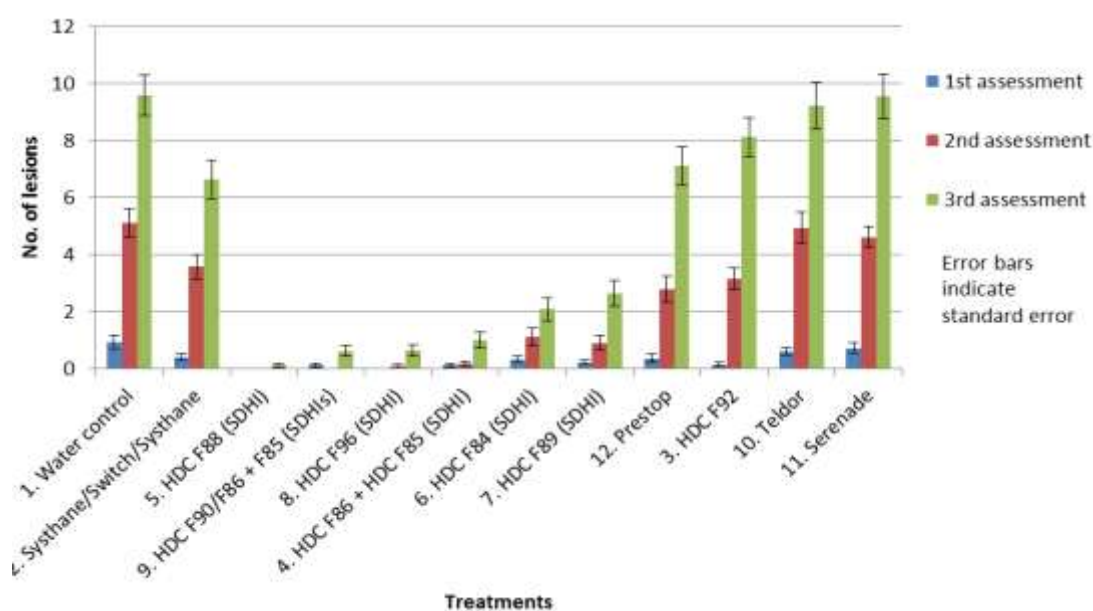
Figure 16. Mean no. of stem lesions per plant caused by *M. melonis* at each assessment date.

At the first assessment, a month after the guard plants were inoculated, the untreated control had the highest number of naturally-occurring lesions (ca. one lesion per plant) on the main stem. No lesions were recorded on plants treated with HDC F88 and HDC F96. The remainder of the fungicide treatments had between 0.1 – 0.3 lesions per plant, with the commercial standard having 0.4 lesions. Plants treated with Teldor and Serenade had only slightly fewer lesions than the untreated control (0.6 & 0.7 per plant respectively).

The lesion number per plant had increased in most treatments by the second assessment, with the exception of HDC F88, HDC F90/F86 + F85 which were free of infection at this stage. Clear differences started to appear between the treatments and four significantly different categories emerged. Plants treated with F86 + F85, HDC F88, HDC F90/F86 + F85 and HDC F96 had the lowest number of lesions per plant (0 - 0.2), followed by plants treated with HDC F84 and HDC F89 (0.9 - 1.1). The products in these first two categories are succinate dehydrogenase inhibitors (SDHIs) in their mode of action, and there was a significant difference between products with this mode of action and the other products used in the trial. The next best treatments were the standard programme, HDC F92 and Prestop with mean stem lesions per plant of between 2.8 and 3.6. The highest numbers of lesions per plant were recorded in plants treated with Serenade, Teldor and the untreated control (4.6, 4.9 and 5.1 respectively).

There was an excellent correlation (R^2 = 0.97) between the results of the second assessment and the results of the third and final assessment. There was also a good correlation between the percentage of stem area with lesions and the total number of lesions on the stem. At the final assessment there were no significant differences in the number of lesions/plant between the untreated and those treated with either the standard fungicide (primarily mildew) programme, Serenade, Prestop, Teldor or HDC F92. A percentage of the plants in each of these treatments had been killed by *Mycosphaerella* stem infections. The percent plant death in the Serenade treatment (25%) was not significantly different from the untreated (32%). The percent plant death in the Prestop treatment (14%) was significantly lower than the percent plant death in the untreated, but this product was generally less effective than many of the experimental fungicide treatments. The percent plant death in the standard treatment (7%), HDC F92 (7%) and Teldor (11%) was not significantly different from treatments with no dead plants. The rest of the treatments were all SDHIs and had significantly fewer lesions per plant than those treatments discussed above. The treatment with the lowest number of lesions per plant was HDC F88 which had only one lesion per ten plants. Plants treated with the SDHI

products F86 + F85, HDC F90/F86 + F85 and HDC F96 had between 0.6 – 1 lesion per plant. Alternating HDC F90 with HDC F86 + F85 did not significantly improve the effectiveness of HDC F86 + F85. HDC F84 and HDC F89 had significantly more lesions per plant (>2) than HDC F88 and 4% of the plants treated with HDC F84 had also died as a result of the infection with *Mycosphaerella*.

Investigating the potential for systemic infection

The inoculation of leaf petioles with mycelial plugs encouraged early development of lesions on the stem, although by the end of the trial the number of lesions per plant was not significantly greater than the number on uninoculated plants due to the rapid rate of secondary spread of the disease. Artificial inoculation of the cut fruit stubs with mycelial plugs dramatically increased the number of stem lesions on inoculated plants.

Inoculation of the main stem wound with both mycelial plugs and conidial suspension increased the incidence of main stem wound infections. Inoculation of the shoot apex with a conidial suspension also increased the incidence of main stem wound infections. This could be due to droplets of conidial suspension falling onto the main stem wound whilst the shoot apex was being spray inoculated. Lesions that developed on the main stem wound were more severe when infections had occurred as a result of conidial inoculations than agar plug inoculations.

The inoculation of the flowers with a conidial suspension led to a higher number of infected fruit on these plants.

Drenching the rockwool block with a conidial suspension did not appear to have an effect on the number of stem lesions. This may be because the plants were too mature by the time this was done and the roots and stem base were therefore not as susceptible to infection by the pathogen. Either way, there was no evidence that such root infection instigated the development of any infection, localized or systemic, within the plant.

Natural infection observed on the leaf petioles increased throughout the trial. No natural petiole infections were observed at the first assessment, but two weeks later between 20% and 70% of the plants in each treatment were infected. By the third assessment the leaf petioles of all plants were infected with the pathogen.

As no fungicides were applied, *Botrytis* became a problem towards the end of the trial. Incidence and part of the plant infected were recorded. *Botrytis* was a secondary pathogen colonizing stem tissues already infected with *M. melonis* and also infected the stems where wounds were present as a result of leaf removal. By the end of the trial more than 50% of

the plants had died as a result of severe *Botrytis* infections. Controlling *M. melonis* would also indirectly control *Botrytis* secondary infections in the crop.

Where plants had been inoculated with a conidial suspension to the shoot tip either on the main stem or laterals a higher incidence of 'burnt out' lateral tips and weak lateral shoots was observed (Figure 17).

The lateral shoots collected from plants which had been inoculated with conidial suspension were compared with those collected from plants which had not been inoculated. In all cases, *M. melonis* was isolated from the nodes on the laterals. This was to be expected as these parts of the plant have more wounds than other plant parts because these are sites
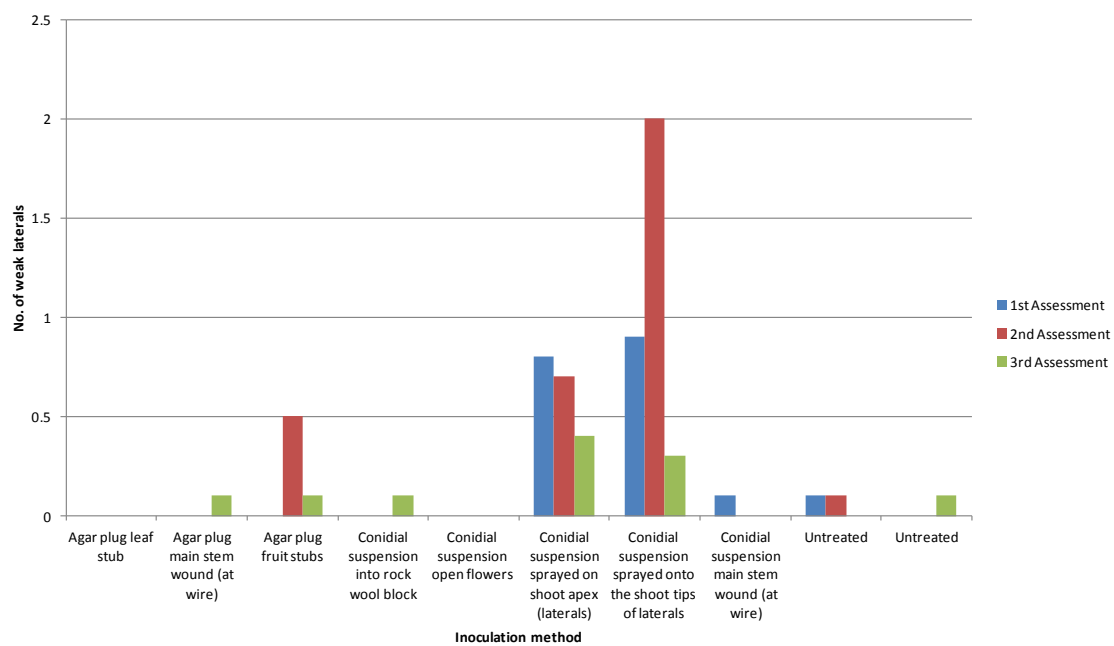
Figure 17. Mean number of weak lateral shoots recorded per plant following various inoculation methods.

where leaves, fruit stubs and side shoots are cut or broken off. In general, though, there were higher numbers of infected nodes on the inoculated laterals than on the uninoculated laterals. In addition a much higher percentage of *M. melonis* was isolated from the internal tissues at nodes and internodes of the inoculated laterals, compared to nodes and internodes of the uninoculated laterals (

Table 5), indicating that the infection could be systemic, rather than superficial as a result of wound infections. However, there was a lot more contamination on plates with the plant pieces from the uninoculated laterals than on the inoculated laterals. Whilst contaminants may have prevented the isolation of *M. melonis* in these cases, the contamination may also have been due to the lack of *M. melonis* present in the stem tissues as it is a fast growing vigorous fungus on agar plates and could potentially inhibit the growth of other

microorganisms. The results certainly support the hypothesis that the pathogen can develop systemically to cause weak, unproductive shoots and from the inoculation data here it suggests that shoot tip infection is the primary route of entry. Further work would be required to confirm these initial results.

Table 5 Comparison between the percentages of *M. melonis* isolated from nodes and internodes of laterals.

Inoculation method	% *Mycosphaerella* recovered from lateral isolations	
	Nodes	**Internodes**
Untreated	12%	8%
Agar plug fruit stubs	23%	6%
Conidial suspension shoot apex	48%	30%
Conidial suspension shoot tips	56%	46%

Conclusions

Evaluation of immunoassay air sampling systems to monitor airborne disease inoculum of *M. melonis* in a glasshouse cucumber cropping system

- From two seasons data collection, results indicate that an ascosporic aerosol concentration of 2000 spores/m^3 of air is required for uniform and severe disease development within a cropping system. Further work in controlled environment conditions would be required to confirm this.

- Initial results would suggest an absorbance value in excess of 0.2 (Low MTIST) and 0.3 (High MTIST) would indicate presence of *M. melonis* at a concentration to initiate disease symptoms on exposed cucumber plants.

- It is unclear whether vertical variation in ascosporic concentration is important with regard placement of the MTIST in the crop and subsequent disease development. Further work is required to determine this.

- Spore sampling identified periods of peak ascospore release occurred between 16.00 and 07.00 hrs.

- If a simple, affordable, spore sampling method were developed for use by growers, effective fungicide applications could be timed to coincide with periods of high infection risk.

Testing of low-risk experimental fungicides and bio-control products

The SDHI fungicides were the most effective at controlling the disease and of these HDC F88, HDC F96 and HDC F86 + F85 performed most effectively. There was, however, slight phytotoxicity observed on plants treated with HDC F88 after the first application. But the product was so effective at controlling *Mycosphaerella* that the application rate could probably be reduced without affecting its efficacy. Later applications when plants were mature caused no such damage. Alternating HDC F90 with HDC F86 + F85 slightly improved the effectiveness of HDC F86 + F85, but HDC F90 was not tested alone, so its' individual effect cannot be evaluated. The products currently approved for use on Cucumber (primarily for powdery mildew control) performed poorly by comparison.

Investigating the potential for systemic infection

The only inoculation technique which produced a significant number of 'burnt out' shoot tips and weak laterals was inoculation of the shoot tips with a conidial suspension of the pathogen. In reality this form of conidial infection may not occur naturally as such spores are sticky and tend to be transmitted to new infection sites by contact rather than air currents. The shoot tips are more likely to be infected by airborne ascospores which adhere to condensed water droplets formed on the tips of the leaves and infect the leaves via hydathodes. Importantly, through aseptic isolation of *Mycosphaerella* from internal shoot tissues on agar it would appear that the pathogen can develop systemically. The data suggests that the infection of the growing tip is the primary entry point for the pathogen. Providing a method could be devised to harvest ascospore inoculum, this work should be repeated to further validate these initial interesting results.

Knowledge and Technology Transfer

5.7.12   Cucumber Growers Association (CGA) meeting at STC. The progress of the project was discussed and the growers were shown the glasshouse trials. In the fungicide trial, they were shown preliminary results of assessments to date which demonstrated the effectiveness of some of the coded products in comparison to the current industry standards. In the systemic infection trial the purpose of the trial was explained and the spore sampling methods and anticipated outcomes were discussed.

19.7.12   Official opening of the LED4CROPS facility at STC. Invited guests were shown the fungicide trial. The purpose of the trial was explained to the audience and they were shown preliminary results of assessments to date which demonstrated the effectiveness of some of the coded products in comparison to the current industry standards.

28.9.12   Technical meeting of the CGA. Powerpoint presentation summarizing the results of the 2012 Fungicide trial at STC.

3.10.12   Cucumber Conference, Peterborough. Presentations:
Disinfectants for control of *Mycosphaerella* in cucumber (Tim O'Neill)
An overview of the epidemiology and control of *Mycosphaerella* in cucumber as a component of HDC Project PE 001a (Martin McPherson)

HDC News Article: Cultural cues to disease control. Issue 188, pp20-21.

References

McPherson, G.M., O'Neill, T., Kennedy, R., Wakeham, A., Wright, K., Goodson, M. and Lambourne, C. (2012). Cucumber – Improving Control of Gummy Stem Blight caused by *Mycosphaerella melonis* (*Didymella bryoniae*). Horticultural Development Company Annual report for project PE 001a.

Van Steekelenburg, N.A.M. (1983). Epidemiological aspects of *Didymella bryoniae*, the cause of stem and fruit rot of cucumber.

Kenedy, R., Wakeham, A.J., Byrne, K.G., Meyer, U.M. and Dewey, F.M. (2012) A new method to monitor airborne inoculum of the fungal plant pathogens Mycosphaerella brassicicola and Botrytis cinerea. Applied and Environmental Microbiology, July 2000. Vol 66, 2996-3000

Appendices

Appendix 1. Fungicide and bio-control trial plan – STC

PE 001a Mycosphaerella control in cucumbers
Fungicide Trial Plan 2012
MFU Zone 6

| Conventional fungicides |
| Biological products |

P A T H

DOOR

cv. Shakira   3 plants/slab   8 plants/plot
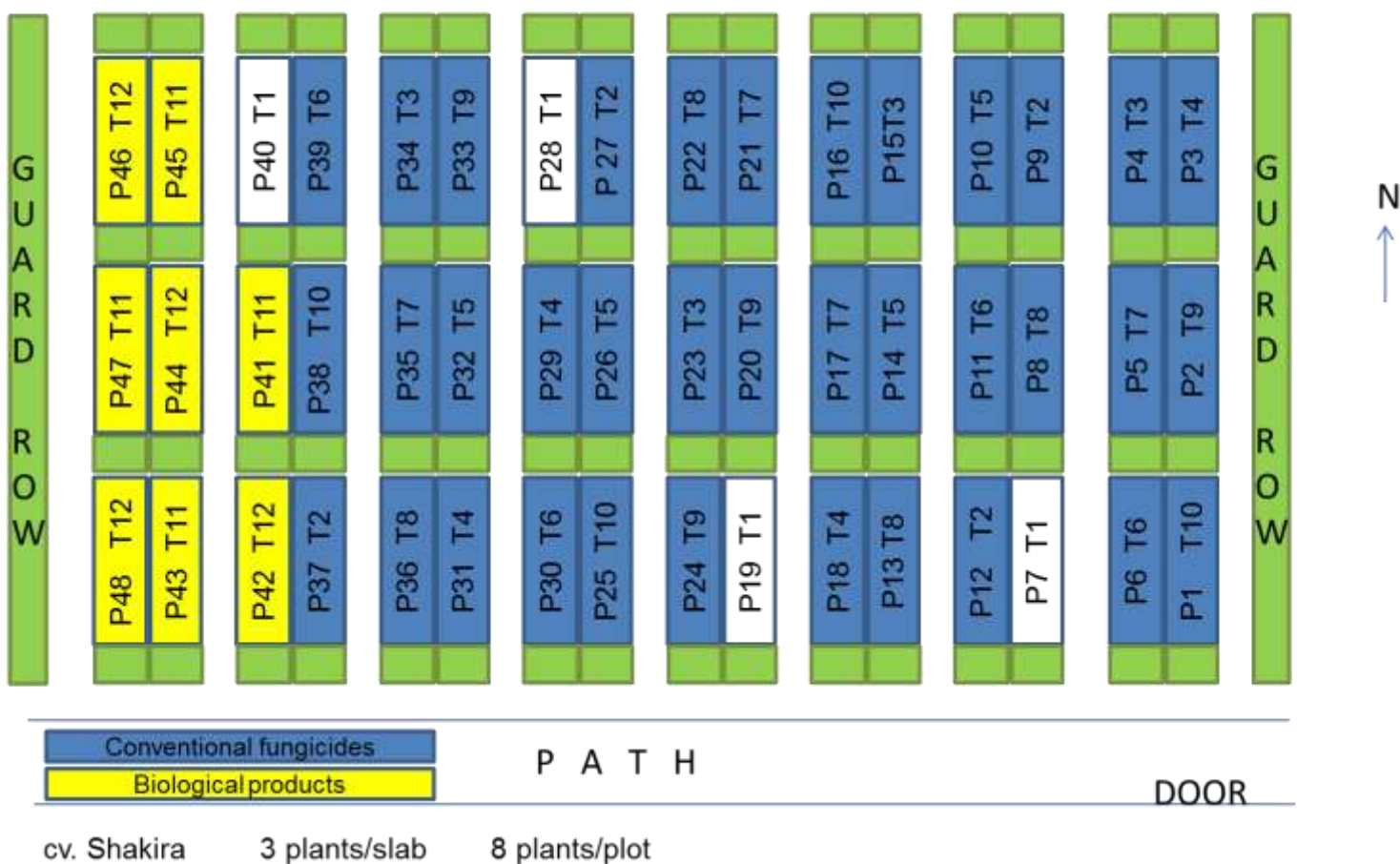
Appendix 2. Systemic infection trial plan

PE 001a *Mycosphaerella* control in cucumbers
Systemic infection Trial Plan 2012
F19 STC

CV. Shakira   2 plants/slab

P93 T8													P8 T3
P94 T5	P92 T1	P79 T5	P78 T10	P65 T6	P64 T2	P51 T9	P50 T5	P37 T8	P36 T10	P23 T1	P22 T4	P9 T8	P7 T5
P95 T9	P91 T3	P80 T8	P77 T2	P66 T3	P63 T5	P52 T7	P49 T2	P38 T6	P35 T3	P24 T8	P21 T6	P10 T1	P6 T7
P96 T6	P90 T9	P81 T2	P76 T4	P67 T1	P62 T8	P53 T4	P48 T7	P39 T1	P34 T9	P25 T2	P20 T2	P11 T5	P5 T10
P97 T4	P89 T6	P82 T10	P75 T7	P68 T9	P61 T10	P54 T1	P47 T9	P40 T4	P33 T2	P26 T9	P19 T9	P12 T3	P4 T6
P98 T2	P88 T1	P83 T7	P74 T3	P69 T4	P60 T6	P55 T5	P46 T1	P41 T8	P32 T5	P27 T7	P18 T4	P13 T10	P3 T9
P99 T7	P87 T3	P84 T4	P73 T6	P70 T7	P59 T3	P56 T2	P45 T4	P42 T6	P31 T7	P28 T5	P17 T6	P14 T7	P2 T2
P100 T10	P86 T5	P85 T8	P72 T9	P71 T1	P58 T8	P57 T10	P44 T10	P43 T3	P30 T3	P29 T10	P16 T8	P15 T1	P1 T4

N

Not inoculated	Inoculated with agar plug	Inoculated with conidial suspension	Door