Project title:	Cucumber – Improving Control of Gummy Stem Blight caused by *Mycosphaerella melonis (Didymella bryoniae)*
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Project leader:	Dr G M McPherson
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** Left STC December 2011*

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

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headlines

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

- A broad range of novel fungicides and bio-control products have been screened in *in vitro* and *in planta* tests and a number of novel products have been demonstrated to have good activity against *M. melonis*.

Background and expected deliverables

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. It causes extensive stem and leaf infections which when severe can debilitate or even kill plants. Air-borne infection of flowers and developing fruit leads to fruit rot. Such infections may become visible in the crop but at other times, probably under specific environmental conditions, this type of infection remains latent (hidden) only developing visually once the fruit has been marketed. These internally infected fruit can sometimes be identified by a tapering to the tip of the fruit though this does not always occur and these latent infections continue to have an economic impact in the industry. They lead to rejection and reduced retailer and consumer confidence in the product. 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 some of the more effective approved fungicides.

An extensive literature review was carried out during Phase 1 of the study. It discussed in detail the pathogen, the disease it causes in cucumbers and the various factors that influence its occurrence, survival, infection and control. The review helped to identify various areas for work on this host/pathogen combination with the work being split into two phases. The expected deliverables from phase 2 of this project 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 of the project and main conclusions

Seed-borne infection

Although the pathogen was suspected at a very low level from work in Phase 1, further extensive testing in 2011 did not find any conclusive evidence of a seed-borne infection route. It therefore seems likely that this route of infection is either absent or very low in current commercial seed stocks. However, as seed-borne infection has been documented previously (Lee *et al*, 1984) growers need to keep alert to the risk, especially when they are trialling small areas of new experimental (numbered) varieties.

Immunoassay spore trap

Work to develop and validate an immunoassay spore trapping system for use on-site by growers and consultants has continued with some promising results. A monoclonal antibody (MAb) to *M. melonis* has been produced following the inoculation of mice with ascospores of the fungus. It has proved insufficiently sensitive and additional work is now being conducted to improve sensitivity. Spore trapping was carried out using two types of samplers in a cucumber crop in Yorkshire over a five month period during 2011. Spores were trapped either on microtitre wells, or on melinex tape, depending on the type of air sampler. Results indicate that spore load is higher low down in the crop and that spore release significantly greater between 17.30 and 03.00 hrs than at other times. This coincides with optimum conditions for infection in the crop when the vents are shut and RH levels are high.

The spore traps are currently being processed using bright field microscopy, which is very time consuming. Once a MAb which is both sensitive and specific has been produced, this can be used to speed up the checking of spore traps. The MAb will also be used to develop a lateral flow test for on-site use to help growers and consultants identify high disease risk periods during cropping. If this alerts them to put control mechanisms into place this should help to reduce severe outbreaks of *M. melonis* arising from ascospore infection.

Novel fungicides and biocontrol products

In Phase 1, some initial laboratory-based studies, using a broad range (29) of isolates of *M. melonis* collected from nurseries in the north and south of England, was carried out. This work checked the current efficacy of approved fungicides (in terms of mycelial inhibition on agar). The work showed that in general mycelial growth of *M. melonis* was inhibited when grown on agar amended with some of the fungicides tested e.g. Teldor (fenhexamid) or by either of the active ingredient components of Switch (cyprodinil & fludioxonil). However,

isolates grown on agar amended with Amistar (azoxystrobin), Bravo 500 (chlorothalonil) or Nimrod (bupirimate) were generally less inhibited. This work was extended substantially in Phase 2 of the study to screen a broad range of novel fungicides (and some bio-control products) for their potential efficacy against *M. melonis*. An initial agar plate screen was conducted and then a second screen was done on young plants using a detached leaf bioassay. A broad range of experimental products (conventional chemicals and bio-control products) were included, listed as coded compounds at the request of the manufacturers and HDC. The identity of the coded compounds will be available when the products become available commercially on the crop.

In the agar plate tests various commercially available and experimental products including Prestop (*Gliocladium catenulatum*), Serenade ASO (*Bacillus subtilis*), HDC F84, HDC F86, HDC F88, HDC F89, HDC F90, HDC F91, HDC F92, HDC F93 and HDC F104 showed potentially good activity against *M. melonis*.

Subsequent tests were carried out on young cucumber plants with a similar range of experimental products (27) and using 2 separate detached leaf bioassays. The tests were carried out following inoculation with two isolates of *M. melonis* (isolated from a northern and southern crop in 2010). In these tests Switch (cyprodinil+fludioxonil), HDC F86, HDC F88, HDC F90, HDC F96 and HDC F98 showed good activity. A short-list of products which showed promise in these bioassays is being taken forward into a large replicated glasshouse study at STC during 2012 (Table 1.)

Table 1. Summary of results from *in vivo* bioassay efficacy testing (2012)

Trt No.	Product	Active ingredient	Reduction in lesion diameter compared to untreated control^	
			Northern isolate	Southern isolate
1	Untreated		-	-
2	Systhane	myclobutanil	***	***
3	Amistar	azoxystrobin	***	**
4	HDC F84	-	***	***
5	HDC F85	-	-	-
6	HDC F86	-	***	***
7	HDC F87	-	**	**
8	HDC F88	-	***	***
9	HDC F89	-	***	***
10	HDC F90	-	***	***
11	HDC F91	-	**	-
12	HDC F92	-	***	***

Trt No.	Product	Active ingredient	Reduction in lesion diameter compared to untreated control^	
			Northern isolate	Southern isolate
13	HDC F93	-	***	***
14	HDC F94	-	***	***
15	HDC F95	-	***	***
16	HDC F96	-	***	***
17	HDC F97	-	*	***
18	HDC F98	-	***	***
19	Switch	cyprodinil + fludioxonil	***	***
20	Teldor	fenhexamid	**	***
21	Nimrod	bupirimate	**	***
22	HDC F99	-	**	***
23	HDC F100	-	*	**
24	HDC F101	-	**	**
25	Prestop	*Gliocladium catenulatum*	**	***
26	Serenade ASO	*B. subtilis*	*	**
27	-	Potassium bicarbonate	*	**

^ based on data from undamaged leaves 5DAT
- No reduction in lesion development compared to the inoculated control.
* represents a slight reduction in lesion development (1-20%)
** represents a moderate reduction (21-60%)
*** represents a good reduction in lesion development (61-100%)

Disinfection

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 2.

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*.

Table 2. 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

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 and hence improve crop yield, marketable quality and hence the economic value of the crop. However, due to the sporadic nature of such pathogen infections it is difficult to put a precise value on this.

Although several fungicide and bio-control products have been shown to provide effective control of *M. melonis* in small-scale laboratory studies, many of these products are not yet approved for use in cucumbers and therefore cannot yet be used commercially. However, the preliminary results help the design of an effective larger glasshouse study conducted during 2012. The results from this work could then be used to recommend additional effective products which may be put forward for approval via SOLA.

If one or more fungicides or bio-control products can be identified and subsequently approved for use on cucumber (with a 1-2 day harvest interval ideally) then significant economic loss could be avoided each year due to premature plant death (from girdling stem lesions) and from symptomatic or latent fruit infections. It is estimated that between 1-10% plants and fruit may be lost as a result of infection by *Mycosphaerella* each year.

It is also worth noting that if a product or products could be found with activity against powdery mildew and *Mycosphaerella* then the financial benefit could be even greater.

It is a little too early to judge the potential financial benefits from the immunoassay work that is in progress but, if the pathogen could be successfully monitored as proposed, then it will help to better time intervention treatments including spray applications and this could provide significant economic benefits in the longer-term through improved disease prediction.

Action points for growers

- Consider using effective disinfectants identified in this project to limit secondary spread of infection during crop work and between crops.

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

- Prestop, Serenade and Switch showed potential efficacy for the control of Mycosphaerella in cucumbers in agar plate and small plant tests and should be considered as part of an effective control regime in commercial crops. A number of experimental or unapproved products also showed promise and may be available for use in the future.

SCIENCE SECTION

Introduction

Gummy stem blight caused by *Mycosphaerella melonis* (*Didymella bryoniae*) has been a persistent leaf, stem & fruit disease in glasshouse cucumber for many years (Fig. 1). 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. This is of considerable concern for growers due to the potential economic damage this pathogen can cause either through direct loss of plants (stem girdling) or yield reduction (as a result of symptomatic or latent (internal) fruit infection). Increased energy costs are a significant factor leading to increased infection as the higher cost discourages the use of pipe heat early in the morning to dry the foliage and avoid conditions conductive 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. 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 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*.

Figure 1. *Mycosphaerella melonis* stem and fruit infection
Picture courtesy of Dr G M McPherson

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. There is a direct parallel here with the use of antibiotics for disease control in human & animal populations and likewise in horticulture we are facing an increased risk of fungicide resistance in phytopathogen populations. 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.

The purpose of this project is firstly to establish 'state of the art' with respect to our knowledge on this important pathogen and to establish the sensitivity of the current population to widely used fungicides (Phase 1). Guided by this knowledge, the aim is then to seek alternative control strategies (Phase 2). This includes the evaluation of novel fungicides & alternative bio-control products and the use of novel immunosassay 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.

Evaluation of disinfectants for activity against M. melonis

Introduction

M. melonis can persist between crops in infected plant debris and possibly as spores contaminating glasshouse surfaces. Within a crop there is potential for dispersal of the fungus by contact transmission on equipment and on the hands of crop workers. Use of disinfectants that reduce inoculum of *M. melonis* should reduce the risk of early infection in a newly planted crop and the rate at which gummy stem blight spreads through a crop. The aim of this work was to identify disinfectants and hand cleansers with good activity against *M. melonis* that could be used as part of a strategy to control the disease. The specific objectives were: to establish the efficacy of a range of chemical disinfectants, from different active ingredient groups, against spores and mycelium of *M. melonis*; to determine the efficacy of some hand cleansers in preventing transmission of *M. melonis*; to determine the efficacy of selected disinfectants for reduction of *M. melonis* on four surfaces (aluminium, concrete, glass, plastic), and to establish the efficacy of some knife-dip disinfection treatment in preventing transmission of *M. melonis* at levels sufficient to cause disease.

Materials and methods

Experiments were carried out in 2011 and 2012 at ADAS Boxworth.

Experiment 1: Efficacy of disinfectants on spores of M. melonis

A culture of *M. melonis* isolated from cucumber in 2010 was incubated at 20°C, on plates of potato dextrose agar + streptomycin sulphate (PDA+S) under alternating periods of 12 h UV lights and 12 h dark until spore-producing pycnidia formed. Plates were flooded with 5 to 10 ml of acidified (pH 3.5 - 4.5), sterile-distilled water (SDW) and a loop was used to scrape the agar surface. A few drops of Tween-80 (wetter) were added to the acidified water in order to increase spore discharge from pycnidia and mitigate spore agglutination. The mixture was filtered through four layers of sterile muslin to remove mycelia, pycnidia, and dislodged agar. A haemocytometer was used to produce a concentration of approximately 1×10^6 spores/ml. The spore suspension was stored at 5°C until use.

Products were tested at the label recommended rate and at half that rate with exposure times of 5 and 30 minutes. For each disinfectant product (Table 1), 10 ml of the spore suspension was pipetted into each of six Universal tubes. For each disinfectant rate (untreated, full rate and ½ rate), requisite volumes were added to the spore suspension in each of two Universal tubes, to give the required concentrations. Five minutes after adding the disinfectant, the two tubes containing the full rate and ½ rate were centrifuged at 2000

rpm for 2 mins. The supernatant fluid was removed and the pellet re-suspended in 5 ml SDW. This was repeated on other tubes of spores after 30 mins disinfectant exposure time, again testing products at the full rate and half rate.

Three replicate 25-well plates of PDA+S were used per treatment, each containing five columns: an untreated control (water), the disinfectant tested at full rate for 5 and 30 mins, and at half rate for 5 and 30 mins; there were five replicate wells for each rate x time combination column. A droplet of the appropriate spore suspension was placed into the centre of each well. The Petri plates were incubated at 20^{o}C and the proportion of wells with visible growth of *M. melonis* was determined after 7 days.

Table 1. Details of disinfectant products used in Experiments 1, 2, 4 and 5.

Product	Active ingredient(s)	Recommended product rate
1. Untreated control	-	-
2. Jet 5	Hydrogen peroxide + PAA	1:125
3. Fam 30	Iodophor	1:125
4. Menno Florades	Benzoic acid	10 ml/L
5. Sodium hypochlorite (10-14%)	Sodium hypochlorite	1:10 [a]
6. Unifect-G	QAC +glutaraldehyde	4%
7. Vitafect	QAC + biguanidine salts	1%

[a] Equates to 10,000 ppm hypochlorite.

Experiment 2: Efficacy of disinfectants on mycelium of M. melonis in filter paper

The same treatments were tested as in Experiment 1. Squares of sterile filter paper (approximately 0.5 cm^2) were cut, and placed on the surface of an actively growing culture of *M. melonis*. The filter paper was left on the cultures for 7 days to allow the fungal mycelium to grow into the paper. The filter paper was then immersed in the disinfectant products at the recommended rate and ½ rate for 5 mins and 30 mins. The filter paper pieces (infested with *M. melonis*) were immersed in SDW as the control treatment. Treated pieces of paper were rinsed three times in SDW, left to dry in the air flow from a laminar flow hood, then plated on to PDA+S in 25-well plates with treatments arranged as described previously for the spore test. The plates were incubated at 20^{o}C and scored on the proportion of wells with growth of *M. melonis* after 7 days.

Experiment 3: Efficacy of hand cleansers against M. melonis

Fingers were contaminated by crushing cucumber fruits naturally infected with *M. melonis* between thumb tip and forefinger tip 10 times. The contaminated thumb tip was applied to a PDA+S plate for 10 successive contacts in paired tests: a) directly and b) after using the hand cleansing treatments (Table 2). Contact was done sequentially from top left to bottom right of the agar plate so that any reduction in transmission with successive contacts would be visible. Hand cleansing was standardized by applying the treatment for 1 minute followed by rinsing under a tap for 10 seconds (direct contact treatments), or allowing the foam sanitiser and hand gel to evaporate for 1 minute. The same thumb and forefinger tip was re-contaminated between treatments by using a fresh piece of cucumber tissue naturally infected with *M. melonis*.

The number of agar plate contacts that developed *M. melonis* after 27 days incubation at 20ºC was recorded. A record was made of the number of transfers to agar that resulted in growth (out of 10).

Table 2. Details of hand cleansing products used in Experiment 3.

Treatment	Active ingredients
1. Untreated control	-
2. Warm water and soap (bar)	Sodium palmate & other salts
3. Cutan Foam Hand Sanitiser	Alcohol
4. Antibacterial Hand Gel	Alcohol

This experiment was repeated using a modified procedure due to a high occurrence of bacteria and yeasts developing at finger contact sites on agar plates in the original experiment which may have affected growth of *M. melonis*.

Mycelium from a 21 day old culture of *M. melonis* on PDA was mixed with internal tissue from a healthy cucumber to form a paste. The paste was rubbed between thumb and forefinger 50 times in order to contaminate fingers in a standard manner. A contaminated forefinger was then applied to a PDA+S plate as described above both immediately and after allowing the paste to air dry. The thumb and forefinger were re-contaminated, allowed to dry and then hands were washed with soap and water (or other hand cleansing test treatment) for 30 seconds; hands were rinsed in tap water, dried on a paper towel and then the contaminated finger was applied to a PDA+S plate. Hands were then washed with soap and water, followed by alcohol gel, before re-contaminating finger and thumb with the *M. melonis* in cucumber paste, and testing another hand-cleansing treatment (Table 3). The

same soap, hand sanitizer and hand gel were used as in the original experiment. Each of the eight treatments was tested three times. Plates were incubated at 20ºC.

The number of agar plate contacts that developed *M. melonis*, and the density of growth of the fungus (0 – nil, 1 – slight, 2 – moderate, 3 – dense), were recorded after 6 and 9 days. Results were examined by regression analysis and analysis of variance.

Table 3. Detail of hand cleansing treatments examined (Experiment 3 revised repeat)

Hand cleansing treatment	Post-cleansing action before touching agar plate
1. None – wet paste	None
2. None – dry paste	Allowed to dry in air
3. Soap and water on dried paste	Water rinse, paper towel dry
4. Foam on dried paste	Allow to evaporate
5. Gel on dried paste	Allow to evaporate
6. Soap and water on dried paste, rinse then foam	Allow to evaporate
7. Soap and water on dried paste, rinse then gel	Allow to evaporate
8. Water rinse only	Paper towel dry

Experiment 4: Effect of surfaces on efficacy of disinfectants against M. melonis

Surfaces of aluminium (glasshouse bench), concrete (pathway), glass (glasshouse wall) and rigid plastic (tray) were initially cleaned by washing in warm water and rinsing with fresh water. They were then contaminated by spaying marked areas (10 x 10 cm) with a suspension of spores and mycelium of *M. melonis* in SDW and allowed to dry for 30 mins.

The contaminated surface was then spray-treated with disinfectant and again allowed to dry for 30 minutes. Each disinfectant in Table 1 was tested at its full recommended rate, and an untreated was included. The treated surface was tested for viable *M. melonis* by swabbing with a new cotton bud moistened in SDW and streaking it over a PDA+S agar plates. Ten swabs were done for each of the 28 disinfectant x surface combinations. The number of swabs that resulted in growth of *M. melonis* after incubation of agar plates for 7 and 14 days at 20ºC was recorded. Results were examined by Generalised linear modeling to determine the effect of surface and disinfectant on transmission of *M. melonis*.

Experiment 5: Practical test – knife treatment to reduce disease transmission

Knife blades were contaminated with *M. melonis* by using them to cut (5 cuts per blade) through cucumber stem and leaf tissue naturally infected with the fungus. The contaminated knife blades were placed in a small container of the test disinfectant at its full rate for 1 hour. The knife blades were then allowed to dry for 15 minutes. Using each knife, three cuts were made across 2-cm thick cucumber slices, to around half the depth of the slice; the same knife was used to cut 10 cucumber slices arranged on damp paper towels in a plastic container. The cucumber slices were incubated at 20ºC in the plastic container

and the proportion of cut slices that developed gummy stem blight was assessed after 5, 7 and 13 days. There were seven treatments (Table 4) with four replicate knife blades for each treatment. Results were examined by generalized linear modeling.

Table 4. Details of treatments used for a practical test on transmission of gummy stem blight (Experiment 5)

Treatment	Rate
1. Tap water	-
2. Jet 5	1:125
3. Menno Florades	10 ml/L
4. Sodium hypochlorite	1:10
5. Vitafect	1%
6. No dip (positive control)	-
7. New knife (negative control)	-

Results and discussion

Efficacy of disinfectants against spores (Experiment 1) and mycelium (Experiment 2) of M. melonis

Results are summarized in Table 5 and illustrated in Figures 1- 2. Differences between treatments were clear and no statistical tests were done. For Experiment 1, all treatment combinations reduced the viability of *M. melonis* spores compared with the untreated control. The Vitafect treatment at half its recommended rate was least effective with 14/15 wells showing growth after 5 and 30 minutes. The Jet 5 and the Fam 30 treatments had greatest efficacy against *M. melonis* spores compared with other treatments, with no wells showing growth in any of the treatment combinations; Sodium hypochlorite and Unifect G were almost as effective.

For Experiment 2, all treatment combinations except those of Menno Florades reduced the viability of *M. melonis* mycelium in filter paper compared with the untreated control. In contrast, *M. melonis* established in most if not all of the wells for all treatment combinations of Menno Florades except when it was used at full rate for 30 mins.

In both experiments Menno Florades showed a fall-off in activity when used at half rate, suggesting that the full rate we used is only just sufficient to kill *M. melonis*. The contrast in the efficacy of Vitafect on spores and mycelium on filter paper at half rate is striking and counterintuitive, as one would expect the filter paper to reduce disinfectant activity. Further work is needed to determine if this is a true difference. When used at full rate however, Vitafect worked well in both tests.

Table 5. Effect of some disinfectant treatments on viability of spores and mycelium of *M. melonis*

Product	Number of wells (of 15) with growth of *M. melonis*			
	Full rate (5 mins)	Full rate (30 mins)	Half rate (5 mins)	Half rate (30 mins)
Spores				
Untreated	15	15	15	15
Fam 30	0	0	0	0
Jet 5	0	0	0	0
Menno Florades	0	0	5	0
Sodium hypochlorite	1	0	0	0
Unifect G	0	1	0	0
Vitafect	1	0	14	14
Mycelium				
Untreated	15	15	15	15
Fam 30	3	0	1	1
Jet 5	0	0	0	0
Menno Florades	13	0	15	14
Sodium hypochlorite	0	0	0	0
Unifect G	0	0	0	0
Vitafect	0	0	0	0

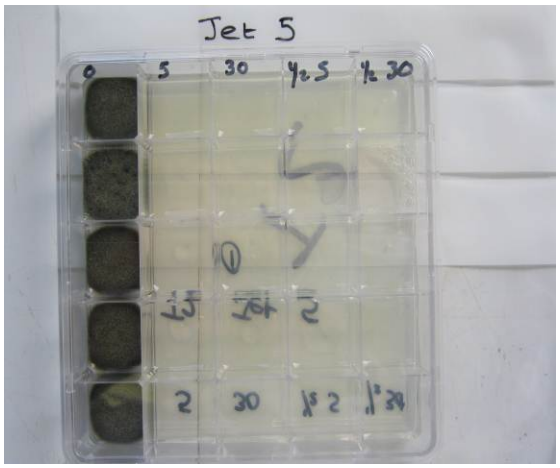


Fig 2.1 Jet 5

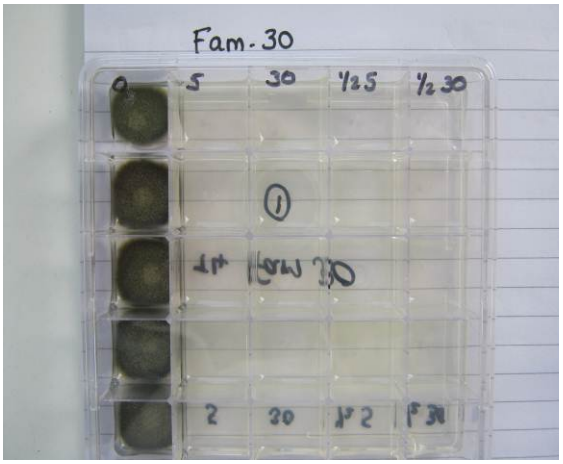


Fig 2.2. Fam. 30

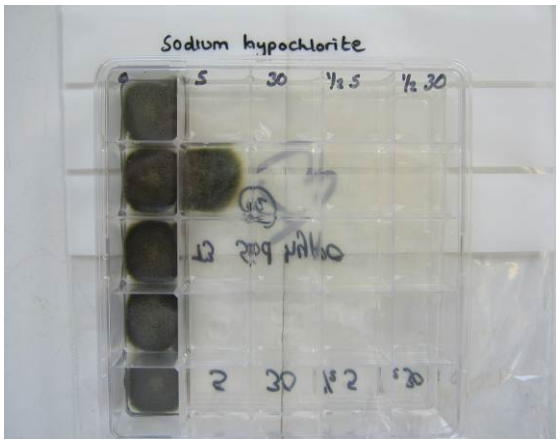


Fig 2.3. Sodium hypochlorite

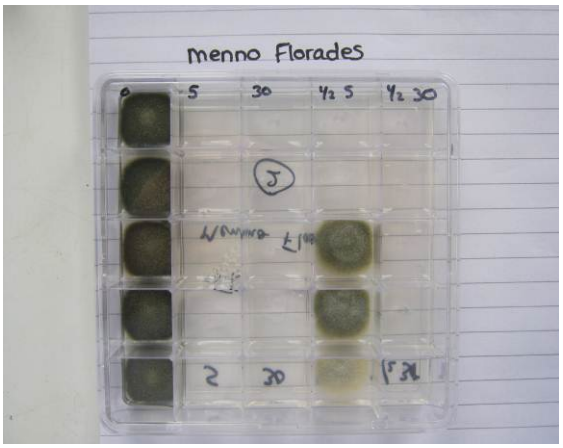


Fig 2.4. Menno Florades

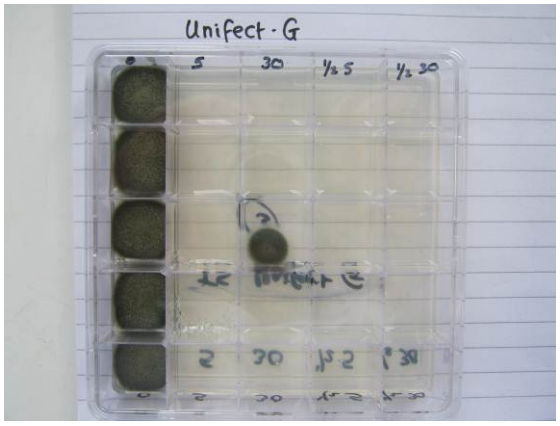


Fig 2.5. Unifect G

Fig 2.6. Vitafect

Figure 2. Effect of six disinfectants on viability *of M. melonis* spores. Treatment columns, from left to right: Untreated; full rate for 5 minutes; full rate for 30 minutes; half rate for 5 minutes; half rate for 30 minutes. Each treatment was tested 5 times (rows within a column) on each of three replicate plates.

Fig 3.1 Jet 5

Fig 3.2. Fam. 30

Fig 3.3. Menno Florades

Fig 3.4. Sodium hypochlorite

Fig 3.5. Unifect G

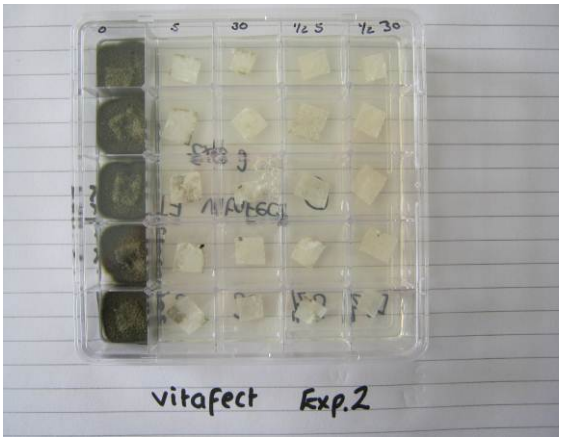


Fig 3.6. Vitafect

Figure 3. Effect of six disinfectants on viability of *M. melonis* mycelium in filter paper. Treatment columns, from left to right: Untreated; full rate for 5 minutes; full rate for 30 minutes; half rate for 5 minutes; half rate for 30 minutes. Each treatment was tested 5 times (rows within a column) on each of three replicate plates.

Experiment 3: Efficacy of hand cleansers

After handling fruit affected by gummy stem blight, growth on agar showed that fingers were contaminated with *M. melonis*, *Penicillium* sp. and bacteria (Table 6). The alcohol foam, alcohol gel and the combined treatment were all effective in reducing levels of *M. melonis* and *Penicillium* recovered from fingers, with the combined treatment resulting in the cleanest plates. There was no evidence of fall-off in transmission with 10 successive contacts on an agar plate.

Table 6. Effect of hand-cleansing treatments on transmission of *M. melonis* and other microorganisms following contamination of fingers with cucumber fruit affected by gummy stem blight – Experiment 3

Treatment	Number of agar plate contacts (of 10) with growth of:		
	M. melonis	*Penicillium* sp.	Bacteria/Yeast
1. Untreated*	8	10	10
2. Soap	0	10	10
3. Foam	0	2	10
4. Gel	1	0	10
5. Foam and gel	0	0	3

* Mean of 4 replicates.

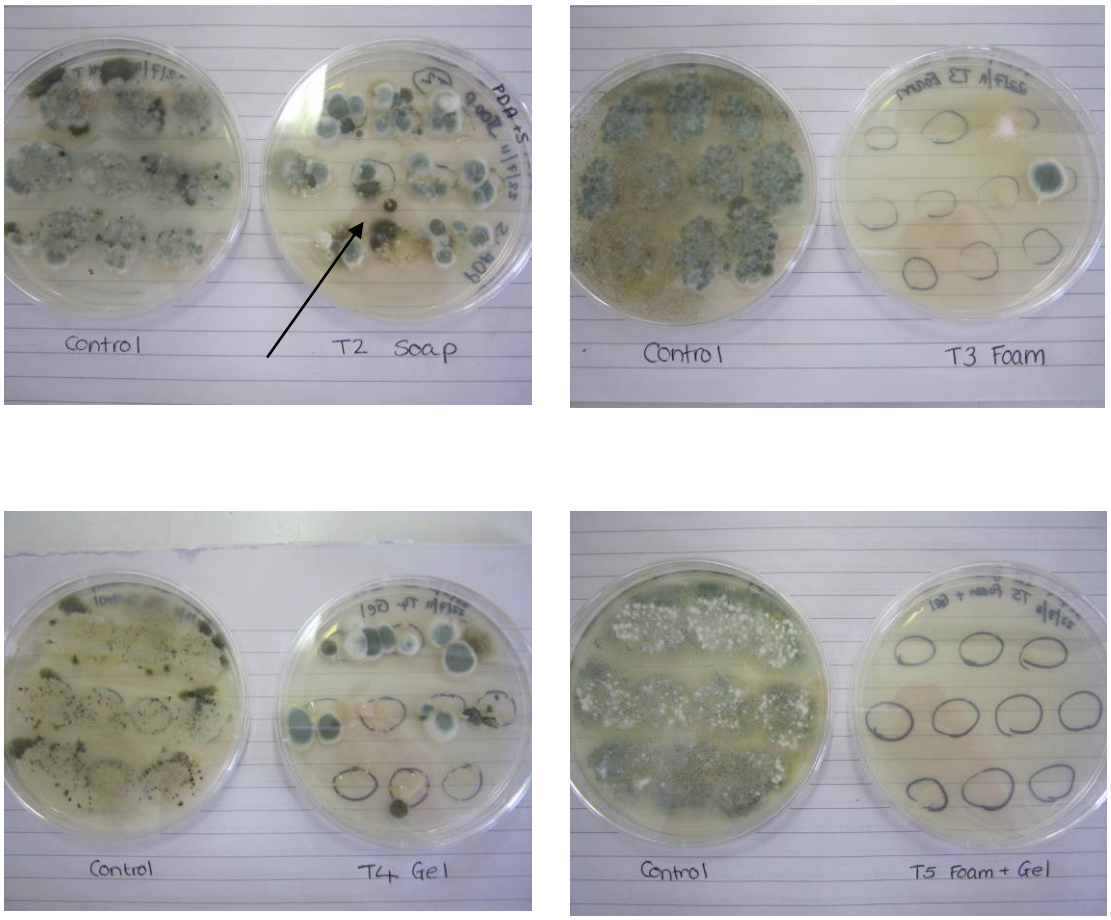


Figure 4. Effect of four hand-cleansing treatments on transmission of *M. melonis* after contamination of fingers with infected fruit. *M. melonis* appears as dark-green to black colonies (arrowed).

In the revised repeat experiment, growth of *M. melonis* on the agar plates from untreated hands was good with considerably less overgrowth from bacteria and *Penicillium* sp. than in the original experiment. The results of assessments at 6 and 9 days were similar; assessment of growth after 9 days is shown in Table 7. Both wet and dry contaminated fingers resulted in growth of *M. melonis* at all contact sites. There was no evidence of fall-off in transmission of *M. melonis* with successive contacts on an agar plate. Transmission of *M. melonis* was significantly reduced ($p < 0.001$) by all of the hand-cleansing treatments that used soap and water and/or alcohol foam or gel; it was not reduced at all simply by rinsing hands in water. The soap and water treatment was significantly ($p < 0.001$) less effective (growth at 27% of contact sites) than treatments that included alcohol foam or gel (growth at 0-7% of contact sites). The mean density of *M. melonis* growth after 9 days was reduced by treatments which reduced transmission of the fungus.

Hand-cleansing treatment also affected transmission of *Penicillium* sp. and bacteria. All treatments were fully effective against *Penicillium* sp. except for rinsing in water. The alcohol foam treatment was most effective at preventing growth of bacteria (Table 7).

Table 7. Effect of hand-cleansing treatments on transmission of *M. melonis* following contamination of fingers with a paste of the fungus in cucumber tissue – Experiment 3 revised repeat, growth after 9 days.

Treatment	Mean % contact sites with growth of:			Density of *M. melonis* (0-3)
	M. melonis	*Penicillium* sp.	Bacteria	
1. None – wet paste	100 (-)	26 (6.1)	73 (8.4)	3.0
2. None – dry paste	100 (-)	23 (5.8)	46 (9.4)	3.0
3. Soap and water	27 (6.8)	0	63 (9.1)	0.5
4. Alcohol foam	0 (-)	0	0	0
5. Alcohol gel	7 (3.8)	0	30 (8.6)	0.1
6. Soap and water; foam	3 (2.7)	0	43 (9.4)	0.1
7. Soap and water; gel	0 (-)	0	13 (6.4)	0
8. Water rinse only	100 (-)	3 (2.5)	10 (5.7)	3.0
Significance (23 df)	<0.001	<0.001	<0.001	<0.001
LSD	-	-	-	0.29

( ) – standard error.

Experiment 4: Effect of four surfaces on disinfectant efficacy

At the rates tested, three disinfectants (Jet 5, sodium hypochlorite and Unifect G), were fully effective against *M. melonis* on all surfaces (Tables 8 and 9). Fam 30 was fully effective on all surfaces except concrete; Menno Florades was fully effective only on glass and rigid plastic. Overall, concrete was significantly more difficult to disinfect of *M. melonis* than glass, plastic and aluminium (Table 10). This may be due to the porous nature of concrete, possibly resulting in entrapment of organic matter that reduced disinfectant activity; or surface tension preventing good contact with contaminated surfaces within pores.

Table 8. Effect of four surfaces on efficacy of various disinfectants against *M. melonis*

Disinfectant	Mean number of swabs (of 10) resulting in growth of *M. melonis* after 7 days on:			
	Aluminium	Concrete	Glass	Plastic
1. Untreated	4 (0.9)	7 (0.9)	6 (0.9)	9 (0.6)
2. Fam 30	0	6 (0.9)	0	0
3. Jet 5	0	0	0	0
4. Menno Florades	1 (0.6)	4 (0.9)	0	0
5. Sodium hypochlorite	0	0	0	0
6. Unifect G	0	0	0	0
7. Vitafect	0	0	2 (0.7)	0

( ) – standard error.

Table 9. Mean effect of various disinfectants against *M. melonis* across four surface types

Disinfectant	Mean number of swabs (of 10) resulting in growth of *M. melonis* after:	
	7 days	14 days
1. Untreated	6.5 (0.4)	6.3 (0.4)
2. Fam 30	1.5 (0.2)	1.5 (0.2)
3. Jet 5	0	0
4. Menno Florades	1.3 (0.3)	1.3 (0.3)
5. Sodium hypochlorite	0	0
6. Unifect G	0	0
7. Vitafect	0.1 (0.02)	0.1 (0.02)

( ) – standard error

Table 10. Mean effect of four surfaces recovery or *M. melonis* across all treatments (including untreated)

Surface	Mean number of swabs (of 10) resulting in growth of *M. melonis* after 7 days
Aluminium	0.7 (1.5)
Concrete	2.3 (0.2)
Glass	1.1 (0.2)
Plastic	1.2 (0.1)

( ) – standard error.

Table 11. Significance of disinfectant and surface on disinfection of *M. melonis* after 7 days

Factor	Df	F probability
Disinfectant	6	< 0.001
Surface	3	< 0.001
Disinfectant x surface	18	< 0.001
Residual	252	
Total	279	

Experiment 5: Practical test - knife treatment to reduce disease transmission

At 5 days after cutting and incubation of cucumber slices, gummy stem blight had developed in the untreated (positive control) and sodium hypochlorite treatment only. By 7 days after cutting, some cucumber slices were infected with *M. melonis* in all treatments except for the negative control (new knife, no contamination with *M. melonis*) and the Menno Florades treatment (Table 12). The incidence of rots compared with the no dip (untreated) control was significantly ($P<0.001$) reduced by simply soaking knives in water for 1 hour, or in Jet 5, Menno Florades, sodium hypochlorite or Vitafect for 1 hour, at the rates used.

After 11 days incubation, gummy stem blight was visible in all treatments including the negative control; this result suggest some infections arose from latent infections of *M. melonis* present on or in the visibly healthy cucumbers used for the tests. This was also evident from the position at which some of the rots originated, arising from the outer surface of the fruit rather than the knife cuts across the centre of fruits. Unfortunately it was not possible to determine the origin of all lesions due to their spread across the fruit sections. At this assessment, the positive control had a much higher incidence of rots (mean 9.3 slices out of 10) than the other treatments (1.8-3.8 slices out of 10), suggesting that most rots originated from the applied knife cuts (Table 12).

Table 12. Effect of knife-dip disinfectant treatments on the transmission of *M. melonis* at levels sufficient to cause gummy stem blight (GSB) in cucumber

Knife dip treatment*	Mean number of cut fruit (of 10) developing GSB after:		
	5 days	7 days	13 days
1. Tap water	0	0.3 (0.2)	2.8 (0.9)
2. Jet 5	0	0.3 (0.2)	3.0 (1.0)
3. Menno Florades	0	0	3.8 (1.1)
4. Sodium hypochlorite	0.3 (0.1)	0.3 (0.2)	1.8 (0.8)
5. Vitafect	0	0.5 (0.2)	2.0 (0.9)
6. No dip (positive control)	2.5 (0.40)	3.0 (0.7)	9.3 (0.6)
7. New knife (negative control)	0	0	1.8 (0.8)
Significance	<0.001	<0.001	<0.001

*Knife blades contaminated with *M. melonis* by cutting through affected tissues were soaked in disinfectant products at the full label rate for 1 hour.

Figure 5. Development of gummy stem blight in cucumber slices cut with a knife contaminated with *M. melonis* (right) compared with a new knife (left).

Development of monoclonal antibody cell lines to ascospore inoculum of *Mycosphaerella melonis*

Introduction

Spore trapping techniques have previously been shown to indicate when pre-symptomatic control of air-borne diseases in the field may be possible e.g. for *Botrytis* blight of onion. However, as different pathogens with differing spore types have varying environmental requirements, accurate differentiated spore counts are necessary. Determining the spore threshold at which infection occurs is also important.

Within glasshouse crop production, monitoring air-borne inoculum concentrations has shown when there is increased risk of disease and where disease control can be targeted. Spore trapping techniques could be used to improve control of gummy stem blight by highlighting periods when there is an infection risk. To achieve this will require the development and validation of an immuno-monitoring system for spores of *M. melonis.*

Knowledge gained from previous work carried out by Dr Roy Kennedy and his colleagues on *Mycosphaerella brassicicola* in field-grown brassica crops has been used as a starting point for this aspect of the project, although work carried out in Phase 1 of this study showed that the monoclonal antibodies developed previously, for *M. brassicicola,* were not sensitive enough for use with *M. melonis* spores and therefore new antibodies have had to be produced.

Materials and Methods

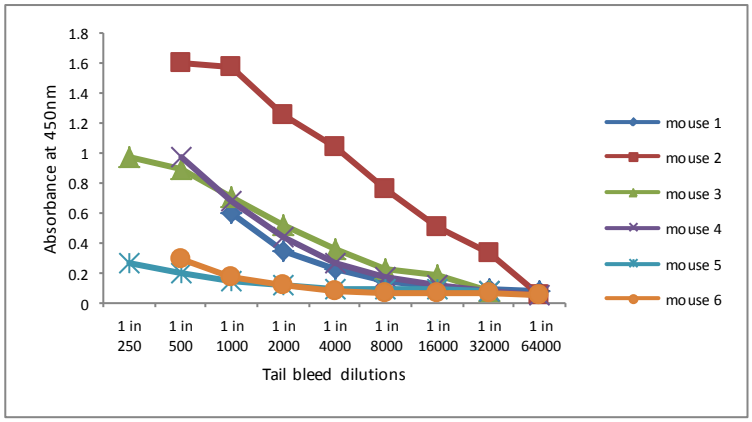
The work was carried out by NAPRU – University of Worcester.

Antibody production

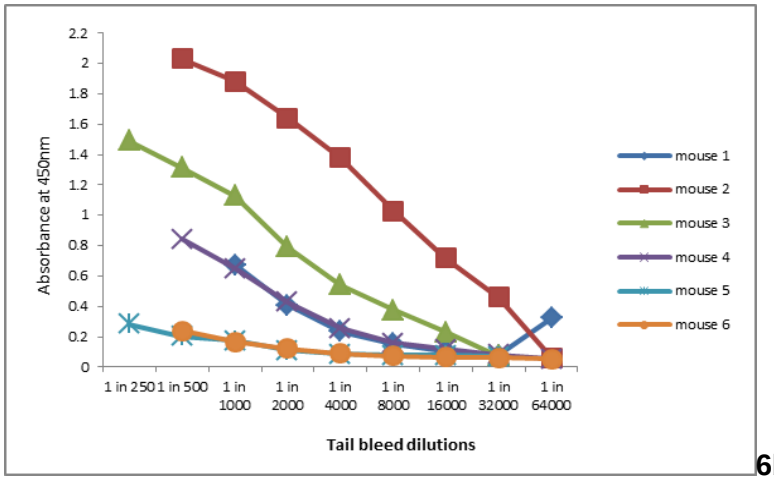
Six mice were immunised with a range of *Mycosphaerella melonis* ascospore fractions:

- Whole ascospore
- Disrupted ascospore fraction > 30 Kda
- Disrupted ascospore fraction< 30 Kda

Immunisations took place on the 28/6/11, 27/7/11 and 23/08/11. Mouse tail bleeds taken on 1/9/11 showed a variable immune response (Figure 6a, b). Immunising with an ascospore fraction of < 30 Kda failed to elicit a good immune response. Two mice have been selected and fusions are underway to identify and select hybridoma cell lines with sensitivity and specificity to ascospores of *M. melonis*.



6a



6b

Figure 6. Mouse tail bleeds - Immune response as recorded by PTA-ELISA to *M. melonis* 'whole' (a) and soluble ascosporic material (b)

Figure 6. Mouse tail bleeds - Immune response as recorded by PTA-ELISA to *M. melonis* 'whole' (a) and soluble ascosporic material (b)

Monitoring glasshouse aerosols for M. melonis

MTIST air sampler.

Two microtitre immunospore traps (MTIST air samplers) were set at variable heights within a commercial cucumber crop in Yorkshire and were operated continuously over a five month period (July to December 2011). The MTIST air samplers operated at a sampling volume of 57 L min^{-1} and air particulates were impacted directly on to the base of 4 x 8 well microtitre strips. To inhibit germination of trapped spora the microtitre wells were pre-coated with 0.05 mg m^{-1}l NaN_3. Following each seven day exposure period the microtitre strips were removed and stored at -20°C prior to analysis by PTA ELISA.

As the weekly Melinex tapes are processed (see Burkard sampler below) the spore counts can be compared to those observed on the weekly MTIST counts. The correlation of these results to-date is quite promising. Spore load was significantly reduced when monitored at 'high canopy' compared to the numbers of spores observed from the air samplers situated low in the canopy.

Commercial crop diary

Aviance crop *in situ* at time of initiation of monitoring

4th July 2011	Bravo application to crop
14th July	Bravo application to crop
25th July	Systhane application to crop
9th August	Crop removal
10th August	Crop replant
11th August	Amistar application
15th August	Bravo application
31st August	Systhane application
15th Sept	Systhane application
4th Oct	Switch/chalk stem spray
5th Oct	Rocket application
18th Oct	Crop removed
2nd Dec	Polythene removed
5-10th Dec	Pressure washed glasshouse
10/11th Dec	Horticide spray space treatment
14th Dec	New polythene laid
22nd Dec	Last despatch of air-sampling tapes and wells.

Figure 7. Burkard (large light green unit at base of crop) and MTIST (two small cylindrical units in upper & lower parts of the crop canopy) air samplers in position at Anchor Nurseries

The PTA ELISA of the glasshouse exposed MTIST wells will be completed on selection of a suitable monoclonal antibody cell lines i.e. immune-quantification of any trapped air-borne ascospores of *M. melonis* in the MTIST wells and this can then be compared alongside the more conventional spore trap results using a Burkard volumetric spore trap (see below).

Burkard volumetric air sampler.

A volumetric air sampler was placed at ground level within a commercial cucumber cropping system and adjacent to an MTIST air sampler (Figure 7). The sampler operated at an air flow rate of 10 L minute^{-1} throughout the five month sampling period. A Melinex tape fixed to a rotating drum and positioned inside the volumetric spore trap, operated continuously for seven day periods, where air particulates in the air were impacted directly onto the tape. At seven day intervals the Melinex tape was removed and sectioned into 24 hr periods. Under bright field microscopy at a magnification of x400, each of the tape sections was examined for the presence of ascospores of *M. melonis* (Figure 8). After which, and on selection of a

suitable MAb cell line the Melinex slides will be processed by immunofluorescence for the presence of *M. melonis* ascospores.

Results

MAb selection

Work is currently on-going to screen and assess the Monoclonal antibodies (MAb). One MAb which has shown good specificity but low sensitivity to *M. melonis* has been identified. This MAb has been used to assess single strips of the weekly MTIST wells (from high and low in the canopy). An additional set of mice have now been immunised and the search for a cell line that will provide both good specificity and sensitivity continues. This will help to make the test transferable to a lateral flow (on-site) test to allow growers/consultants to monitor *M. melonis* spore loads in the cropping area.

Spore trapping of *M. melonis*

The bright field counting of air-borne ascospores of *M. melonis* is currently in progress and results for a short period during September 2011 is presented at Figure 8 below. The recovery of moderate to high numbers of air-borne ascospores during this period using the conventional spore trapping technique is encouraging. The immune-fluorescence of the exposed Melinex tape sections (identification of trapped *M. melonis* by labelling with monoclonal antibody antiserum and visualisation by fluorescein dye) will commence as soon as a suitable monoclonal cell line is identified. Due to the nature of the Burkard air sampler it was possible to determine the time period when the majority of the spores were released. In general the spores released on the dates shown in Figure 8 were released between 17.30 and 03.00 and this information may well be linked to diurnal rhythms of the fungus which ensure spores are released when environmental conditions are most conducive to infection.

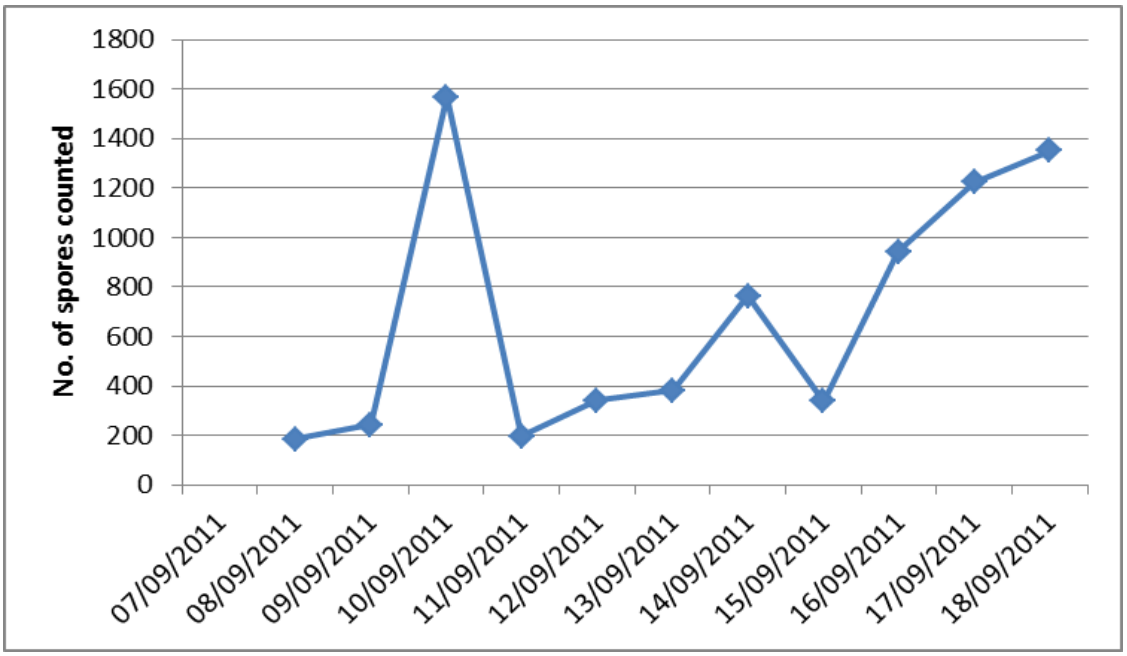


Figure 8 Burkard volumetric bright field counts of airborne *M. melonis* in a commercial cucumber crop during a two week sample period.

Seed testing

Introduction

The extensive literature review carried out in Phase 1 of this study suggested that although there is some compelling evidence of *M. melonis* being seed-borne in some cucurbit crops, extensive research has found the pathogen only sporadically in cucumber seed. Lee *et al* (1984) reported that of over 90 cucurbit seed (cucumber & pumpkin) samples tested from thirteen countries, nine from four countries were found to be infected with *Didymella bryoniae (M. melonis)*. The pathogen was reported to be located on and in the seed coat including the perisperm and in the tissue of the cotyledons. Primary seedling infection occurred on the radicle, hypocotyl and cotyledons. Infection of the radicle generally caused a pre-emergence rot while infection on the hypocotyl and cotyledons developed further inoculum for infection of the first true leaves and the stem.

A small-scale investigation into the potential for *M. melonis* to be seed-borne was carried out during phase 1 of this study. At that time five seed batches were tested and *M. melonis* was detected on two of these, due to the significance of these findings it was agreed that these batches would be retested along with additional batches of different cultivars from a range of suppliers.

Methods & materials

A total of 16 cucumber seed batches were obtained for additional testing at STC during 2011. Re-testing of a retained sample of the cultivar which had given a positive result for *M. melonis* when testing in 2010 was also carried out. Each batch was visually examined under a low power microscope to check for fungal growth on the seeds or debris mixed in with the seed which might prove to be particles of infectious material such as pycnidia. Any suspicious material found was removed and cultured on a suitable artificial growth media to determine its identity. A batch of 100 seed/cv were then plated aseptically onto multiple agar plates (square 25 well plates were used to avoid the potential for contamination to grow across Petri-dishes) which were incubated at 23°C for 7-10 days in order to be able to identify any fungal contaminants on or in the seed. A further batch of 25 seeds was sown in fresh compost (Levington F2+S) in half seed trays and allowed to germinate and grow on for 2-3 weeks. The seedlings were then examined for any visual signs of infection before being excised to allow a microscopic examination of the vascular tissues to take place. Stem slices (2mm) were then cut from each stem and plated onto artificial growth media for

incubation. The stem slices were then examined for the development of any fungal growth which was identified and recorded.

Results

Seed testing was carried out on a total of 17 batches of seed during 2011. The results of the tests are summarised in Table 13 below. One batch (E528/55) that tested positive for *M. melonis* in 2010 was found to be free of *M. melonis* in 2011. It is unclear whether the 2010 result was a false positive (mis-identification or contamination) or, if true, whether the level and viability of the fungus had declined to zero by the re-test. None of the seedlings grown for 3 weeks from these seed batches developed symptoms of gummy stem blight. Microscopic examination of stem vascular tissue plated out onto an agar medium for the presence of *Mycosphaerella* and other potential cucumber pathogens was negative and no plant pathogens were detected from the vascular tisues.

Table 13 . Results of the seed-testing carried out during 2010 and 2011

Seed batch code	When tested	*M. melonis* detected from seed plating (no. of infected seed)	Systemic *M. melonis* detected (stem sections)
E528/S1a	Sept 2010	0	Not tested
E528/S1b	April 2011	0	0
E528/S2a	Sept 2010	0	Not tested
E528/S2b	May 2011	0	0
E528/S3a	Sept 2010	0	Not tested
E528/S3b	April 2011	0	0
E528/S4a^	Sept 2010	1	Not tested
E528/S4b	April 2011	0	0
E528/S5*	Sept 2010 & May 2011	3 0	Not tested 0
E528/S6	May 2011	0	0
E528/S7	April 2011	0	0
E528/S8	May 2011	0	0
E528/S9	May 2011	0	0
E528/S10	April 2011	0	0
E528/S11	May 2011	0	0
E528/S12	May 2011	0	0
E528/S13	April 2011	0	0
E528/S14	May 2011	0	0
E528/S15	April 2011	0	0
E528/S16	May 2011	0	0
E528/S17	May 2011	0	0

* re-tested due to significant result ^ not enough seed left for re-testing
S#a and S#b signify different batches of the same cultivar

No evidence of *Mycosphaerella* was detected on the surface or internal tissues of the additional batches of seed tested using the agar plate test during 2011, or on the re-test of batch E528/S5. None was detected in the growing-on tests in any batches tested.

Secondary screen of novel fungicide and bio-control products for efficacy against *M. melonis*

Introduction

This aspect of the work aims to identify potential products following the loss of key fungicides which has resulted in growers experiencing increasing problems with control of gummy stem blight in cucumbers. Pressure is mounting to reduce the use of conventional chemical products in edible crops and it is therefore important to also evaluate alternative bio-control strategies that could help maintain effective control without recourse to frequent chemical application. It should be remembered that pathogens such as *Mycosphaerella* are one of the reasons for the industry repeatedly re-plant crops each season, and this has cost implications which effective disease control mechanisms may help to reduce.

Materials & Methods

This aspect of the work was carried out in two stages at STC in North Yorkshire.

A primary screen of 20 conventional fungicides and 3 bio-control products was carried out in the laboratory using an *in-vitro* agar plant assay. The details of these products are shown in Table 14 below. The potential efficacy of the products was tested using an amended agar plate test which provides quantitative data on the inhibition of fungal growth (mycelium only) when grown on agar plates amended with each of the products and compared to growth of the fungus on un-amended agar plates. Each product was added to a standard fungal agar medium – Potato Dextrose Agar (PDA) at 2, 20 and 100ppm of the active ingredient in the case of the conventional fungicide products and at the label dilution rate for the bio-control products (Prestop & Serenade). Two previously collected isolates of *M. melonis* were used in the tests, one which had been collected from infected crops in the north of England (Humberside) and the other from a southern crop (Lea Valley) during the 1st year of the study and which had been retained in the STC culture collection. Each isolate was grown on PDA to ensure purity of the culture and tests were set up using 5-7 day old cultures. The sterile PDA was amended with the product under test and poured into Petri-dishes before being allowed to set. Once set, a 5mm plug of the actively growing isolate was positioned centrally on the agar plates. Each isolate was tested against each product, at each concentration, in triplicate.

Table 14. Details of products used in *in vitro* fungicide and bio-control screen - 2011

Trt No.	Product	Active ingredient	Rate/ha	rate/L
1	Untreated	-	-	-
2	Systhane 20EW	myclobutanil	0.375L	0.37ml
3	Amistar	azoxystrobin	1L	1ml
4	HDC F84	-	1L	1ml
5	HDC F85	-	1L	1ml
6	HDC F86	-	0.5kg	0.5g
7	HDC F87	-	0.3kg	0.3g
8	HDC F88	-	0.25L	0.25ml
9	HDC F89	-	1.5L	1.5ml
10	HDC F90	-	0.4L	0.4ml
11	HDC F91	-	1.5L	1.5ml
12	HDC F92	-	0.9L	0.9ml
13	HDC F93	-	0.3L	0.3ml
14	HDC F94	-	1 L	1ml
15	HDC F103	-	0.71kg	0.71g
16	Teldor	fenhexamid	0.1kg/100L	1g
17	Nimrod	bupirimate	0.2L/100L	2g
18	HDC F99	-	0.25L	0.25ml
19	HDC F101	-	0.25L	0.25ml
20	Unix*	cyprodinil	-	1.35g
21	Bravo 500^{+}	chlorothalonil	-	2.0ml
22	Prestop	*Gliocladium catenulatum*	3.5% v/v	35g
23	Serenade ASO	*B. subtilis*	10L	10ml
24	-	Potassium bicarbonate	-	I molar

* Rate chosen to match ai content in Switch

The test plates were incubated at 23°C for 3 days before the diameter of the fungal growth was recorded. The mean colony diameter for each product/concentration/isolate was calculated and compared to the growth of each respective isolate on un-amended PDA. The percentage inhibition of growth was calculated using the following formula.

$$100 - \left(\frac{[mean\ test\ diameter]}{[mean\ control\ diameter]} * 100 \right)$$

In the 2nd phase of the efficacy screening a similar range of 27 products were chosen and tested *in planta* (Table 15). Young plants cv. Femspot were raised in rockwool blocks in the glasshouse. When the plants had reached the 3-4 true leaf stage of growth the products were applied using a hand sprayer (Hozelock Ltd) until leaf wetness was achieved. All products were used at the manufacturers recommended rate. The leaves were allowed to dry before the 2nd true leaf was removed from 6 plants for each product tested. The detached leaves were bagged immediately and returned to the laboratory where 7cm discs were cut from each leaf. The leaf discs were placed on filter paper, pre-moistened with sterile distilled water, in sterile Petri-dishes. The leaves were then 'inoculated' with a 5mm agar plug of an actively growing *M. melonis* culture. The tests were set up on undamaged leaf discs and leaf discs damaged by cutting the leaf surface below the plug position using a sterile scalpel and, as with the previous *in vitro* tests, a northern and southern isolate of the pathogen was used. Six replicate leaf discs/product were used. Leaves from untreated plants were used as a positive control.

The inoculated leaves were assessed by measuring the diameter of the lesion formed on each leaf disc. Any potential phytotoxicity following product application was also recorded. This initial detached leaf bio-assay was assessed after 5 days and again 4 days later (5 and 9 DAT). A second bio-assay was carried out 13 DAT by removing the 3rd true leaf (which had received the same spray treatment) and repeating the tests described above. It was hoped that this would give some indication of the longevity of any protectant activity or systemic properties of the products applied.

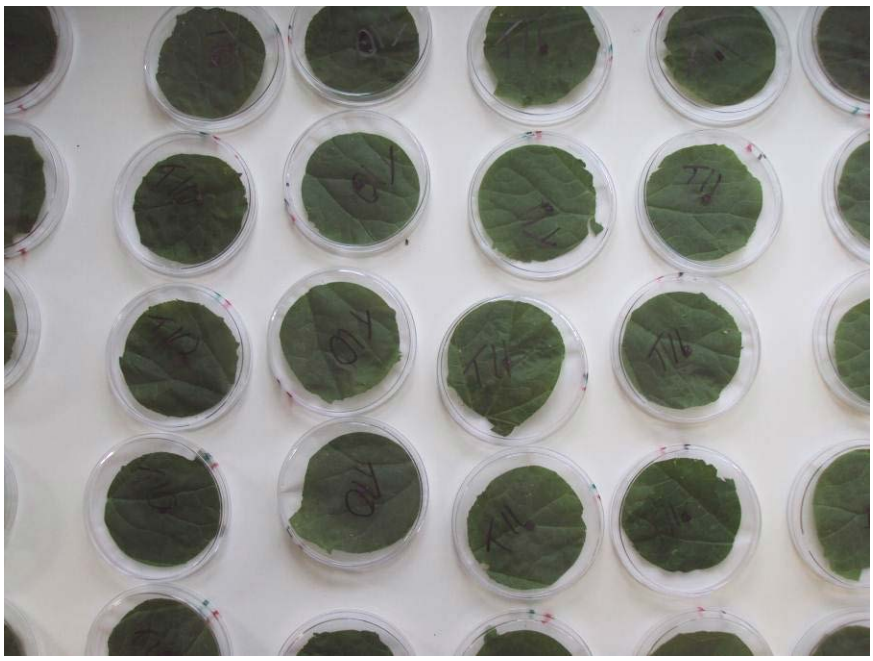


Figure 9. Cucumber leaf discs used in detached leaf bio-assay

Table 15. Details of products and rates used in detached-leaf bio-assay - 2011

Trt No.	Product	Active ingredient	Rate/ha	Rate/L
1	Untreated			
2	Systhane	myclobutanil	0.375L	0.37ml
3	Amistar	azoxystrobin	1L	1ml
4	HDC F84	-	1L	1ml
5	HDC F85	-	1L	1ml
6	HDC F86	-	0.5kg	0.5g
7	HDC F87	-	0.3kg	0.3g
8	HDC F88	-	0.25L	0.25ml
9	HDC F89	-	1.5L	1.5ml
10	HDC F90	-	0.4L	0.4ml
11	HDC F91	-	1.5L	1.5ml
12	HDC F92	-	0.9L	0.9ml
13	HDC F93	-	0.3L	0.3ml
14	HDC F94	-	1 L	1ml
15	HDC F95	-	0.02kg/10L	2g
16	HDC F96	-	0.8L	0.8ml
17	HDC F97	-	0.71kg	0.71g
18	HDC F98	-	0.9L	0.9ml
19	Switch	cyprodinil + fludioxonil	0.8kg	0.8g
20	Teldor	fenhexamid	0.1kg/100L	1g
21	Nimrod	bupirimate	0.2L/100L	2g
22	HDC F99	-	0.25L	0.25ml
23	HDC F100	-	0.125L	0.125ml
24	HDC F101	-	0.25L	0.25ml
25	Prestop	*Gliocladium catenulatum*	3.5% v/v	35g
26	Serenade ASO	*B. subtilis*	10L	10ml
27	-	Potassium bicarbonate		1 molar

Results

In vitro product efficacy screen

The results for each isolate are shown in Appendix 2, however here the two values have been averaged to give an overall indication of the potential efficacy of the products to inhibit mycelial growth.

At the lowest concentration of active ingredient (2ppm) several of the products did not inhibit mycelial growth to any great degree (Figure 10). However, several of the products, including all the experimental products, Serenade ASO and Prestop amongst others did result in appreciable levels of inhibition in this test.

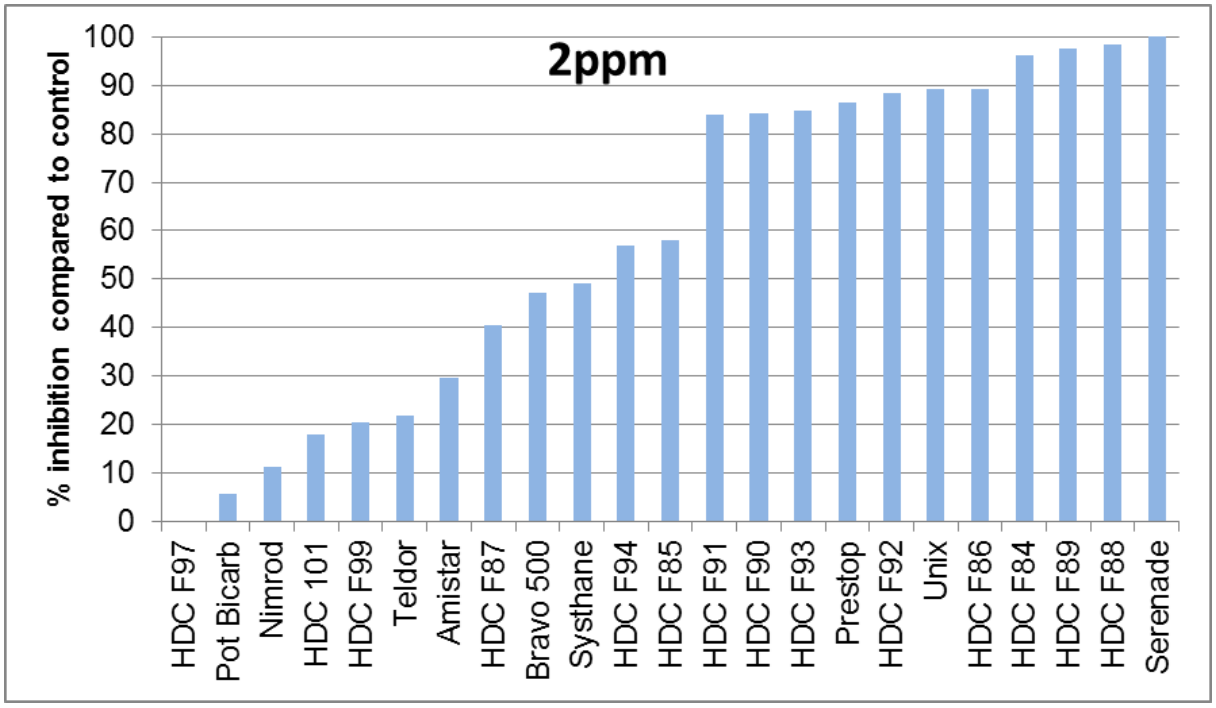


Figure 10. Percentage inhibition of mycelial growth of each product compared to the growth of the fungus on un-amended agar (negative control) for products tested at 2ppm (mean of north & south isolate results).

More of the products under test achieved a higher level of inhibition of growth when used at 20ppm of active ingredient. Nimrod, Teldor, Systhane, HDC F85 and HDC F94 all showed an increase in inhibition at this concentration (figure 11). Products such as Potassium bicarbonate, HDC F87, HDC F99, HDC F101, Bravo 500 and Amistar were less effective resulting in <60% inhibition of growth in these tests.

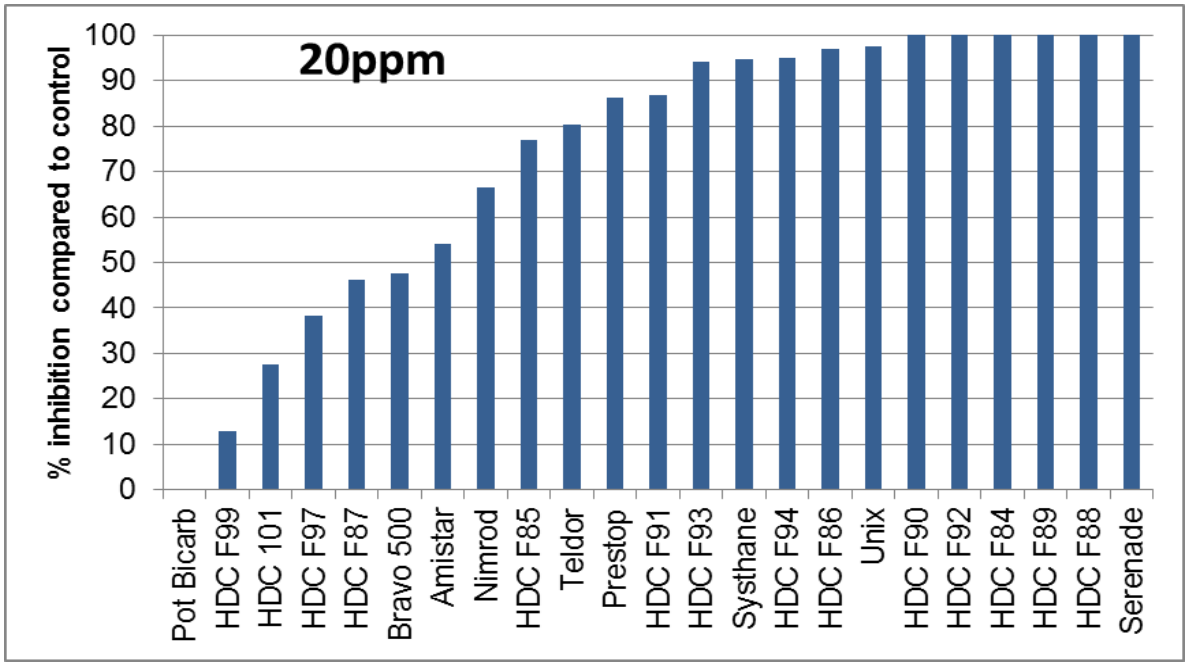


Figure 11. Percentage inhibition of mycelial growth of each product compared to the growth of the fungus on un-amended agar (negative control) for products tested at 20 ppm (mean of north & south isolate results).

When the isolates were grown on agar amended with the products at 100ppm of active ingredient all but 5 of the products resulted in >60% inhibition of growth of the fungus (figure 12). Potassium bicarbonate, HDC F87, HDC F99, HDC F101 and Amistar were still the poorest of the products tested, Bravo 500 and HDC F103 increased their inhibition activity slightly, whilst 50% (11) of the products resulted in 100% inhibition of growth.

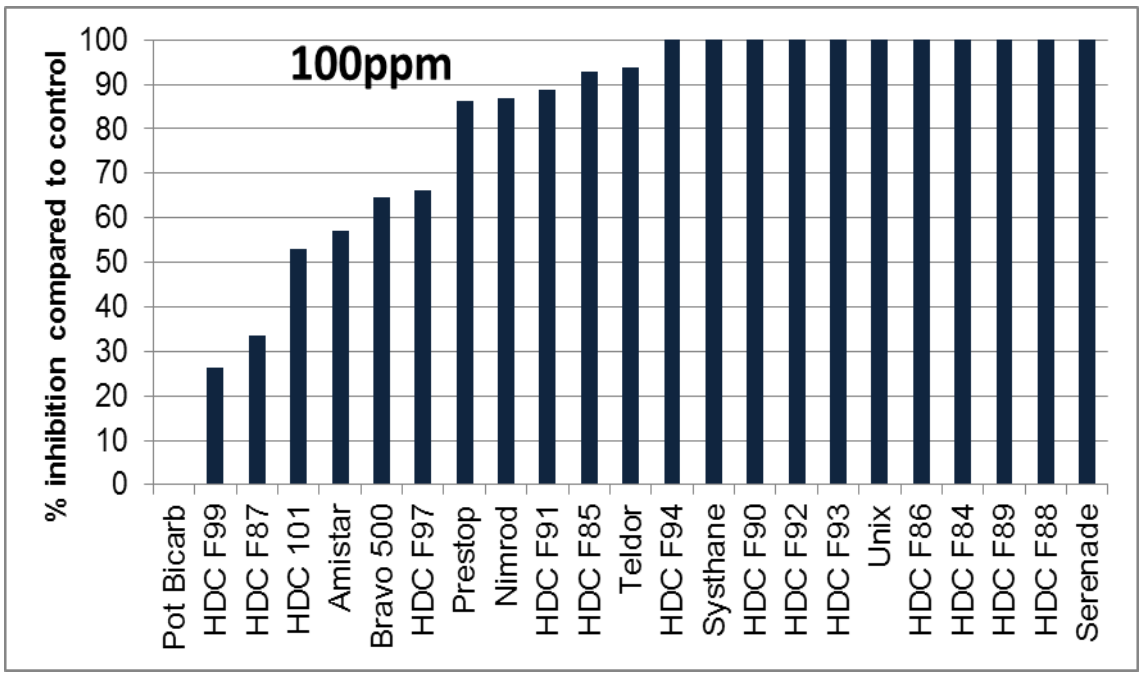


Figure 12. Percentage inhibition of mycelial growth of each product compared to the growth of the fungus on un-amended agar (negative control) for products tested at 100 ppm (mean of north & south isolate results).

It should be borne in mind that due to the nature of this *in vitro* test it only provides a preliminary impression of the potential efficacy of the products to control the fungus. It is not definitive as some products are likely to have a different mode of action other than inhibition of mycelial growth e.g. disruption of spore production, and this would not be measurable in this type of test. Therefore there is a risk that such products would appear to perform poorly and therefore be excluded prematurely and this needs to be considered carefully when drawing any conclusions from such work. However, as a stand-alone test the initial results are encouraging and suggest that several experimental products have potential activity against *Mycosphaerella*.

In planta product efficacy screen

Data was recorded separately for each isolate, and any evidence of a potential phytotoxicity effect from the treatments was recorded at each assessment date. The mean values (across replicates) are shown in tables 16 & 17 and figures 14 & 15 show the overall (averaged) picture of the north and south isolate response.

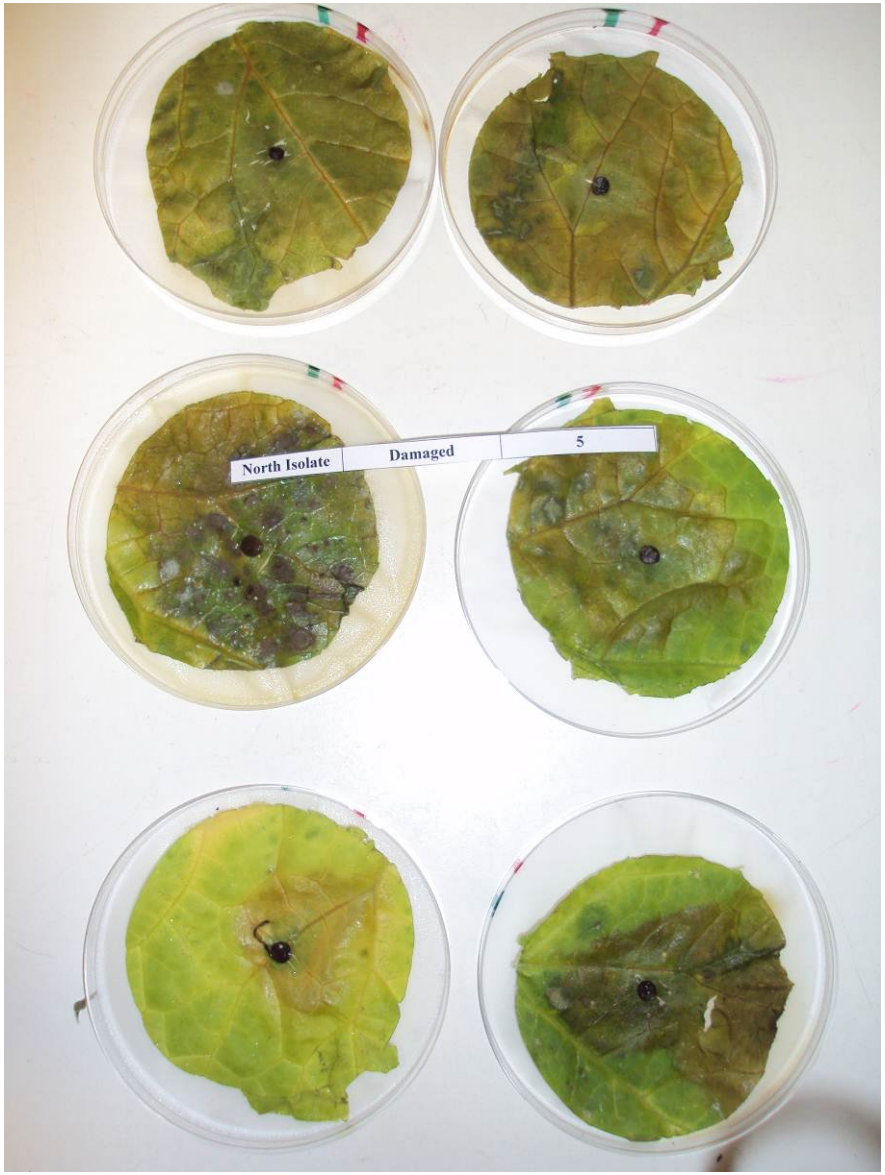


Fig 13a. *Mycosphaerella* lesions developing on leaf discs T5 (HDC F85).

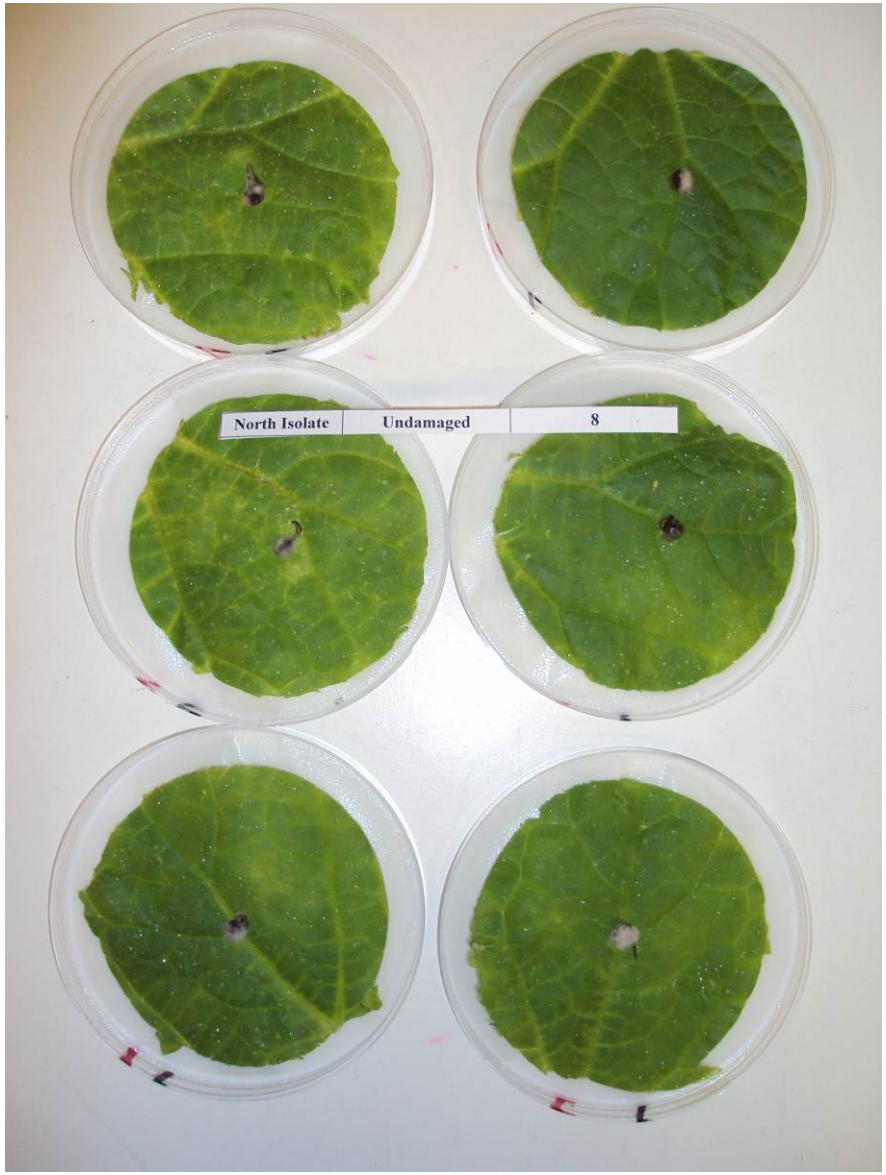


Fig 13b. Leaf discs treated with T8 (Exp.2) showing no lesion.

Table 16. Detached leaf bio-assay results for the northern isolate. Results shown as the % inhibition of lesion development compared to the untreated control (T1)

Treatment	UNDAMAGED			DAMAGED			Possible phytotoxicity effects observed
	Bio-assay 1		Bio-assay 2	Bio-assay 1		Bio-assay 2	
	5 DAT	9 DAT	13 DAT	5 DAT	9 DAT	13 DAT	
2. Systhane	No data – no growth on untreated leaves		13.27	73.91	46.75	28.26	
3. Amistar			24.02	81.52	85.70	23.55	
4. HDC F84			15.64	78.80	64.99	21.94	
5. HDC F85			35.48	-5.98	-219.53	34.05	
6. HDC F86			100.00	97.28	68.93	97.04	
7. HDC F87			51.54	48.37	-76.04	25.71	
8. HDC F88			98.88	97.83	83.23	97.58	
9. HDC F89			25.42	95.11	57.59	37.42	
10. HDC F90			100.00	100.00	96.55	99.60	
11. HDC F91			39.53	29.89	-24.26	39.43	
12. HDC F92			81.98	94.57	85.70	51.82	
13. HDC F93	83.67	78.02	18.75	78.44	83.31	15.92	
14. HDC F94	85.71	74.83	42.42	99.54	97.03	2.92	Leaf edges chlorotic
15. HDC F95	95.41	43.62	78.41	89.91	68.74	-31.03	
16. HDC F96	100.00	96.81	99.24	99.54	98.30	90.98	Leaf edges chlorotic
17. HDC F97	-2.04	-3.36	-0.76	13.30	23.76	-61.27	
18. HDC F98	100.00	94.30	99.43	100.00	95.05	72.15	Stunting and rolling of leaf edges
19. Switch	90.31	79.03	71.78	100.00	89.82	85.68	
20. Teldor	-13.78	-7.21	-6.44	54.59	47.38	-47.75	
21. Nimrod	65.31	10.07	-7.20	50.92	17.68	-30.50	
22. HDC F99	18.88	21.81	NO DATA	32.11	47.24	NO DATA	Severe leaf chlorosis
23. HDC F100	-5.61	0.17	15.72	16.51	8.35	-49.60	
24. HDC F101	18.37	-7.92	-5.49	41.28	8.06	-68.17	
25. Prestop	17.35	3.86	-6.63	54.59	36.78	-63.66	
26. Serenade	22.45	-8.39	11.36	6.88	-1.84	-66.84	
27. Pot bicarbonate	0.51	-8.56	-8.14	9.63	6.79	-62.33	

Table 17. Detached leaf bio-assay results for the southern isolate. Results shown as the % inhibition of lesion development compared to the untreated control (T1)

Treatment	UNDAMAGED			DAMAGED			Possible phytotoxicity effects observed
	Bio-assay 1		Bio-assay 2	Bio-assay 1		Bio-assay 2	
	5 DAT	9 DAT	13 DAT	5 DAT	9 DAT	13 DAT	
2. Systhane	100.00	100.00	-36.58	70.37	65.57	49.45	
3. Amistar	35.62	84.03	-58.35	46.30	77.05	96.15	
4. HDC F84	96.42	100.00	64.37	83.33	93.44	41.76	
5. HDC F85	-168.24	-88.19	80.21	-51.85	-127.05	-9.34	
6. HDC F86	100.00	100.00	98.02	100.00	100.00	100.00	
7. HDC F87	100.00	100.00	93.07	35.19	77.87	98.90	
8. HDC F88	100.00	100.00	100.00	98.15	97.54	100.00	
9. HDC F89	100.00	100.00	45.57	100.00	72.95	98.90	
10. HDC F90	100.00	100.00	100.00	100.00	95.08	100.00	
11. HDC F91	-57.37	70.14	100.00	-33.33	10.66	36.26	
12. HDC F92	71.39	100.00	40.62	85.19	94.26	82.42	
13. HDC F93	97.26	97.17	78.69	100.00	98.92	No data – no growth on untreated leaves	
14. HDC F94	94.52	85.84	97.54	100.00	90.32		Leaf edges chlorotic
15. HDC F95	100.00	100.00	79.91	97.17	50.54		
16. HDC F96	100.00	87.73	99.59	98.11	97.85		Leaf edges chlorotic
17. HDC F97	82.18	85.84	38.92	91.51	84.95		
18. HDC F98	100.00	100.00	100.00	100.00	100.00		Stunting and rolling of leaf edges
19. Switch	100.00	100.00	100.00	100.00	98.92		
20. Teldor	73.96	72.63	78.28	93.40	91.40		
21. Nimrod	94.52	74.52	29.09	90.57	46.24		
22. HDC F99	61.62	23.56	NO DATA	93.40	-35.48		Severe leaf chlorosis
23. HDC F100	41.06	15.06	-26.66	40.56	7.53		
24. HDC F101	20.50	-33.07	84.83	58.49	40.86		
25. Prestop	54.77	61.31	66.39	73.58	46.24		
26. Serenade	58.88	51.87	90.16	55.66	18.28		
27. Pot bicarbonate	43.80	59.42	93.44	51.88	37.63		

A comparison of the data shown in Tables 16 &17 shows that some of the products resulted in a consistent and comparable response between the two isolates, yet the results for other products are very variable between the isolates, with a moderate/good response with one isolate and a complete lack of efficacy in the other. It is difficult to explain the discrepancy in the results here and therefore the data should be treated with caution.

Damaged leaves did not appear to be more susceptible to infection with the fungal isolates than undamaged leaves in general and, as the virulence of these isolates was already proven, this was not unexpected. Products can be characterised by being effective as contact products e.g. showing inhibition of lesion development very quickly following application, how persistent they are, and also whether they have any systemic activity e.g. the product is translocated around the treated plant. It may be expected that most products, where effective, would show some control of lesion development 5 and 9 days after treatment application and this was determined during the 1st bioassay. To determine whether any of the products under investigation also demonstrated longevity of activity, or possibly had systemic activity a 2nd bioassay was carried out 13 days after treatment application. Table 18 shows the product responses compared in this way, and also helps to highlight the different responses observed between the isolates, and therefore where potentially unreliable data may have been gathered. Several of the products used showed good efficacy with both isolates and also as contact and systemic products e.g. HDC F86, HDC F88, HDC F90, HDC F96, HDC F98 and Switch (cyprodinil+fludioxonil). Products such as HDC F85 and HDC F91 were perhaps more effective as systemic products, although the results for these products were less consistent across the two isolates.

The tables of results show that possible phytotoxicity symptoms were observed on the plants following application of HDC F94, HDC F96, HDC F98 and HDC F99. This information is important and if any of these products are taken forward into a larger study this aspect of their performance will require careful monitoring.

Table 18. A comparison of isolate and efficacy responses observed during the detached leaf bioassays

Treatment	North Isolate				South Isolate			
	Contact response^		Systemic response		Contact response^		Systemic response	
	Undamaged	Damaged	Undamaged	Damaged	Undamaged	Damaged	Undamaged	Damaged
2. Systhane	No data	***	*	**	***	***	-	**
3. Amistar		***	**	**	**	**	-	***
4. HDC F84		***	*	**	***	***	***	**
5. HDC F85		-	**	**	-	-	***	-
6. HDC F86		***	***	***	***	***	***	***
7. HDC F87		**	**	**	***	**	***	***
8. HDC F88		***	***	***	***	***	***	***
9. HDC F89		***	**	**	***	***	**	***
10. HDC F90		***	***	***	***	***	***	***
11. HDC F91		**	**	**	-	-	***	**
12. HDC F92		***	***	**	***	***	**	***
13. HDC F93	***	***	*	*	***	***	***	No data
14. HDC F94	***	***	**	*	***	***	***	
15. HDC F95	***	***	***	-	***	***	***	
16. HDC F96	***	***	***	***	***	***	***	
17. HDC F97	-	*	-	-	***	***	**	
18. HDC F98	***	***	***	***	***	***	***	
19. Switch	***	***	***	***	***	***	***	
20. Teldor	-	**	-	-	***	***	***	
21. Nimrod	***	**	-	-	***	***	**	
22. HDC F99	*	**	No data	No data	***	***	No data	
23. HDC F100	-	*	*	-	**	**	-	
24. HDC F101	*	**	-	-	*	**	***	
25. Prestop	*	**	-	-	**	***	***	
26. Serenade	**	*	*	-	**	**	***	
27. Pot bicarbonate	*	*	-	-	**	**	***	

- No reduction in lesion development cf control,
* slight reduction in lesion development (1-20%)
** moderate reduction in lesion development (21-60%)
*** good reduction in lesion development (61-100%)
^ based on results at 5DAT assessment

Contact response – leaves inoculated within 2 h of spray application.
Systemic response – leaves inoculated 13 days after spray application

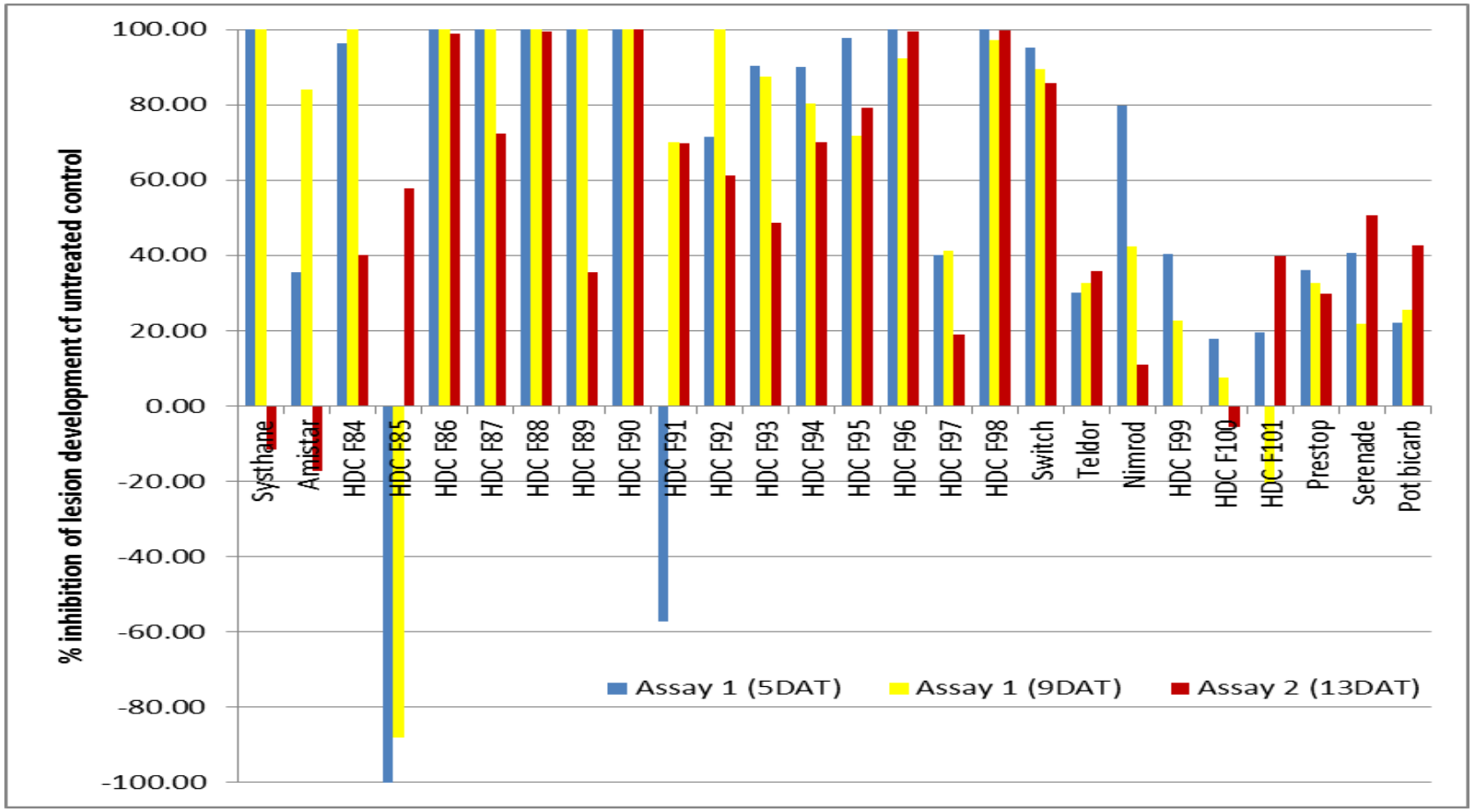


Figure 14. Percentage reduction of lesion size observed in detached leaf bioassay on undamaged leaves (mean of north & south isolate results).

NB – Mean negative values capped at -100% in this chart

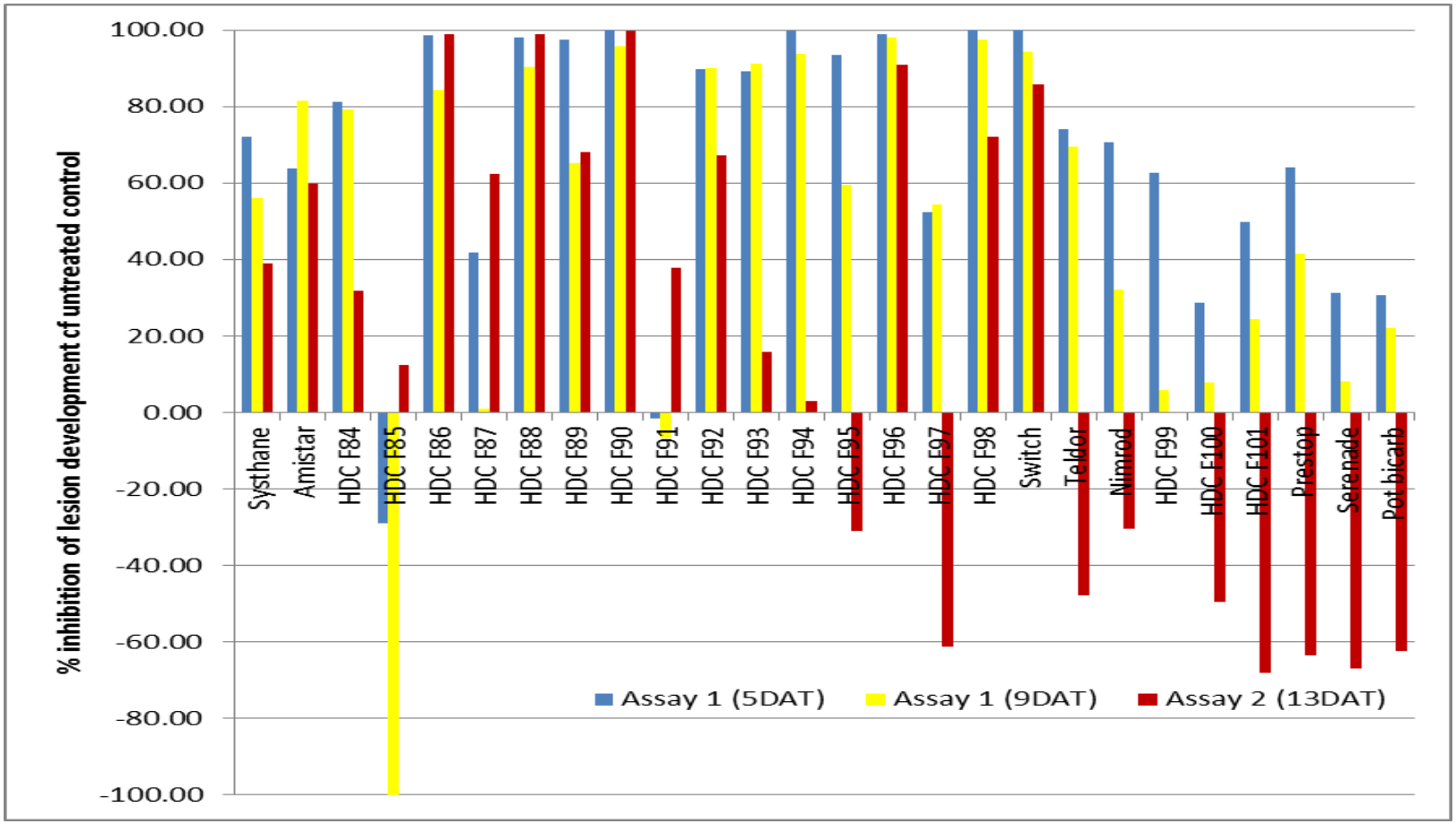


Figure 15. Percentage reduction of lesion size observed in detached leaf bioassay on damaged leaves (mean of north & south isolate results).

NB – Mean negative values capped at -100% for graphing purposes

Conclusions

Disinfectants

1. Six disinfectants tested at their full rate; (Fam 30 at 1:125; Jet 5 at 1:125; Menno Florades at 10 ml/L; 10-14% sodium hypochlorite at 1:10; Unifect G at 4% and Vitafect at 1%) were fully effective against spores of *M. melonis* in water. When tested at half-rate, all products were fully effective except for Menno Florades (effective at 30 mins but not after 5 mins) and Vitafect (not effective after 5 or 30 mins).
2. Four disinfectants tested at their full rate (Jet 5, sodium hypochlorite, Unifect G and Vitafect) were fully effective against mycelium of *M. melonis* in filter paper after 5 mins. Fam 30 and Menno Florades were effective after 30 mins.
3. On surfaces contaminated with a suspension of *M. melonis* spores and mycelium, concrete was more difficult to disinfect than aluminium, glass or plastic. Jet 5, sodium hypochlorite and Unifect G were fully effective on all four surfaces; Fam 30 on concrete, Menno Florades on aluminium and concrete and Vitafect on glass all showed reduced activity.
4. In a practical disease transmission test, soaking knives contaminated with cucumber sap and *M. melonis* for 1 hour in water, Jet 5, Menno Florades, sodium hypochlorite or Vitafect appeared to reduce development of gummy stem blight in cucumber fruit.
5. Dry hands contaminated with a paste of *M. melonis* in cucumber sap resulted in transmission of the fungus when placed on an agar culture plate.
6. Washing hands with an alcohol foam or gel was more effective than soap and water in reducing transmission of *M. melonis* from hands onto a culture medium. Rinsing hands in water did not reduce transmission of the fungus.

Immunoassay spore detection

1. Spore trapping carried out at a commercial nursery during 2012 has provided information on the diurnal periodicity (daily cycle or rhythm) and positional effects of spore release. Significantly more spores were found to be released lower in the crop and peak times for release were between 17.30 and 03.00.
2. A monoclonal antibody cell line to ascospores of *M. melonis* was produced. However, it is not as specific or sensitive as would be desired and further work to select a better antibody is in progress.

Seed testing

1. No conclusive evidence of seed infected with *M. melonis* was observed during this study. However, previous research has proven this to be a potential source of infection and therefore both seed providers and growers should remain alert to the possibility of this risk especially on new or experimental cultivars.

Fungicide screening

1. The fungicide screening work identified a number of potential new products with efficacy against *Mycosphaerella*. The results from the agar plate tests suggest that products such as HDC F84, HDC F86, HDC F88, HDC F89, HDC F90, HDC F91, HDC F92, HDC F93, HDC F104, Prestop, and Serenade may all be worth further investigation in larger glasshouse studies.
2. The detached leaf bio-assays confirmed some of these findings (for products such as HDC F86 and HDC F88 & HDC F90), but also identified other products which performed better *in planta* than *in vitro* e.g. HDC F96, HDC F98 and Switch. Other products gave promising, but inconsistent results.
3. These tests have clearly identified some good potential products for further work and consideration, and have also identified some products that have no efficacy against this pathogen and can therefore be dropped from further studies e.g. Potassium bicarbonate.

Knowledge and Technology Transfer

The results from Phase 1 of the work were discussed at an HDC Project Review Meeting at Stoneleigh on the 21st January 2011. Dr Martin McPherson also presented the results to the Cucumber Growers Association meeting on the 1st February 2011. Dr McPherson gave a detailed update on the project to the industry at the Annual Cucumber Growers Association Conference on the 5th October 2011.

References

Lee D H; S B Mathur & P Neergaard (1984). Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedlings of cucumber and pumpkin. *The Danish Government Institute of Seed Pathology for Developing Countries, Copenhagen, Denmark Phytopath. Z.* **109,** 301-308.

Appendix 1 Crop diaries for disinfectant work

Experiment 1. Efficacy of disinfectants on spores of *M. melonis*

Trial Task	Date completed
Culture *M. melonis* from culture collection slope	13/05/2011
M. melonis placed under UV light	16/05/2011
Experiment plated out: inoculated with $2.5x10^5$ spores/ml	13/06/2011
7 day assessment	20/06/2011
Experiment cleared up and plates discarded	20/06/2011

Experiment 2. Efficacy of disinfectants on mycelium of *M. melonis* in filter paper

Trial Task	Date completed
Subbed on plates of *M. melonis*	01/06/2011
Experiment plated out: mycelium and filter paper	15/06/2011
7 day assessment	22/06/2011
Experiment cleared up and plates discarded	22/06/2011

Experiment 3. Efficacy of hand cleansers

Trial Task	Date completed
Experiment set up: hand cleansers	22/07/2011
7 day assessment	29/07/2011
27 day assessment	19/08/2011
Revised repeat experiment:	
Experiment set up: hand cleansers	17/01/2012
6 day assessment	23/01/2012
9 day assessment	26/01/2012

Experiment 4. Effect of four surfaces on disinfectant efficacy

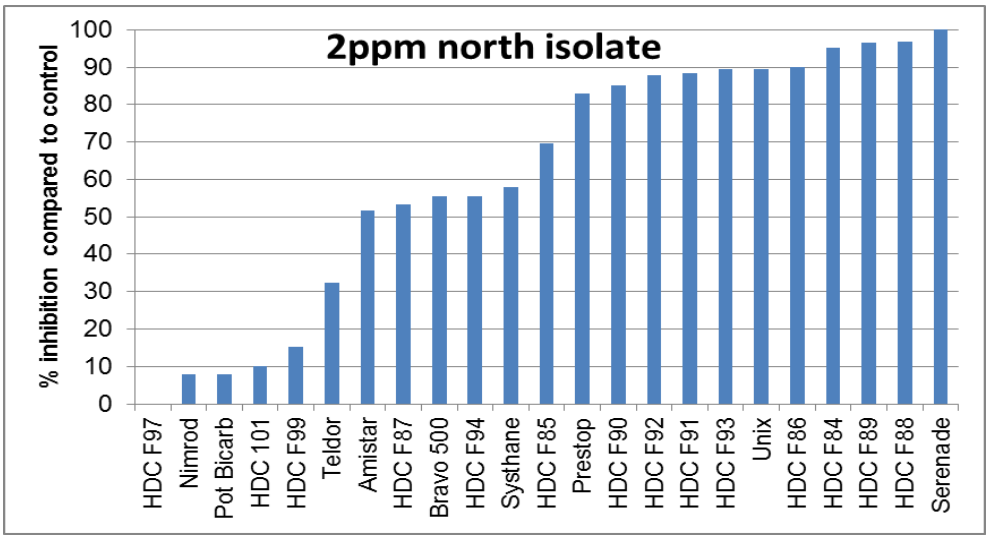
Trial Task	Date completed
Surfaces cleaned and then sprayed with *M. melonis* spore suspension	25/08/2011
Surfaces sprayed with relevant disinfectant after 30 minutes	25/08/2011
Surfaces swabbed after 30 minutes and streaked onto agar	25/08/2011
7 day assessment of agar plates	01/09/2011
14 day assessment of agar plates	15/09/2011

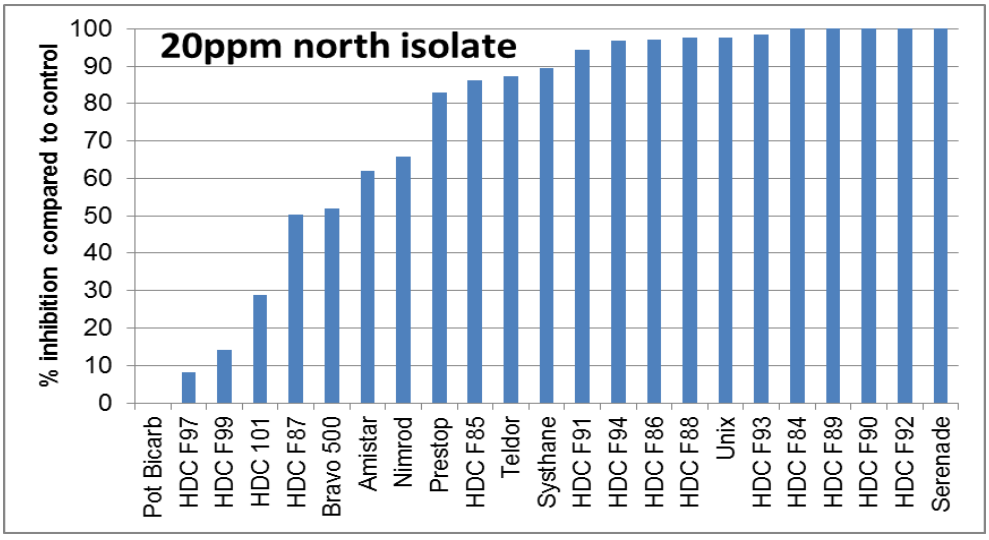
Experiment 5. Practical test- knife dip treatment to reduce disease transmission

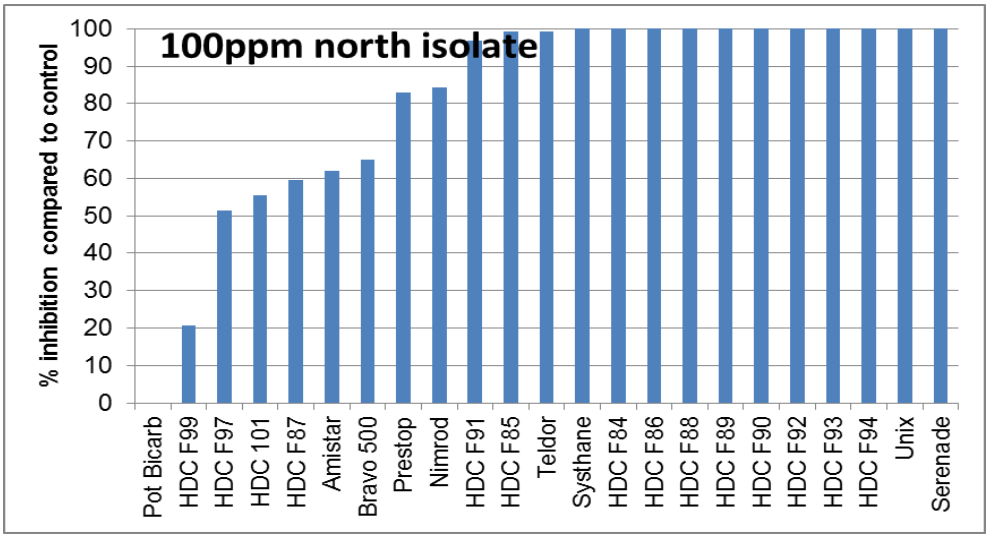
Trial Task	Date completed
Preliminary experiment 1 set up (to check decay of slices)	16/05/2011
3 day assessment of cucumbers in preliminary experiment	19/05/2011
7 day assessment of cucumbers in preliminary experiment	23/05/2011
10 day assessment of cucumbers in preliminary experiment	26/05/2011
14 day assessment of cucumbers in preliminary experiment	30/05/2011
Preliminary experiment 2 set up (to check decay of slices cut with a contaminated knife)	22/07/2011
3 day assessment of cucumbers in preliminary experiment	25/07/2011
7 day assessment of cucumbers in preliminary experiment	29/07/2011
Experiment set up	19/08/2011
5 day assessment	24/08/2011
7 day assessment	26/08/2011
14 day assessment	02/08/2011

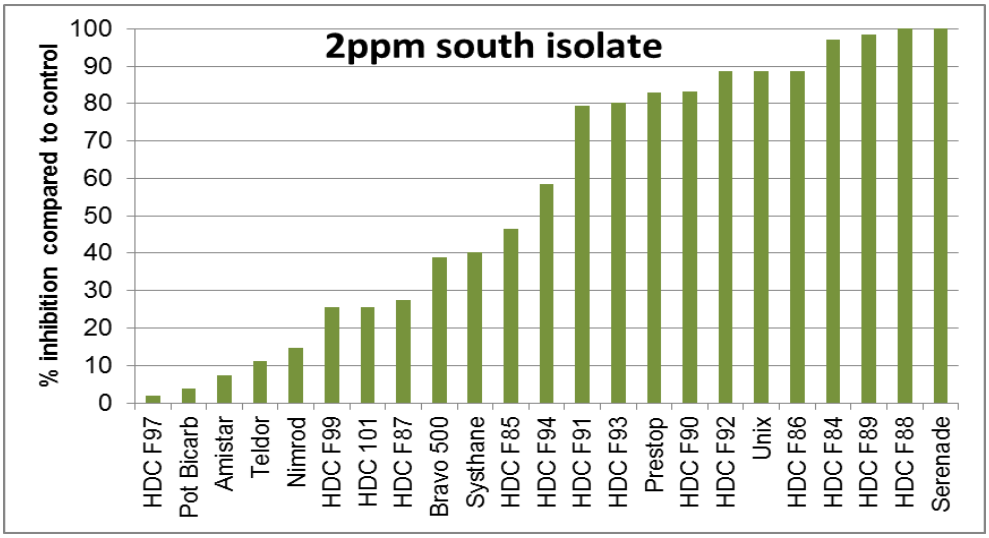
Appendix 2. In vitro *product screening results*

Charts showing results for the isolates of *M. melonis* collected from the north and south nurseries during the *in vitro* (agar plate) product screen.








2ppm south isolate
% inhibition compared to control
100
90
80
70
60
50
40
30
20
10
0
HDC F97
Pot Bicarb
Amistar
Teldor
Nimrod
HDC F99
HDC 101
HDC F87
Bravo 500
Systhane
HDC F85
HDC F94
HDC F91
HDC F93
Prestop
HDC F90
HDC F92
Unix
HDC F86
HDC F84
HDC F89
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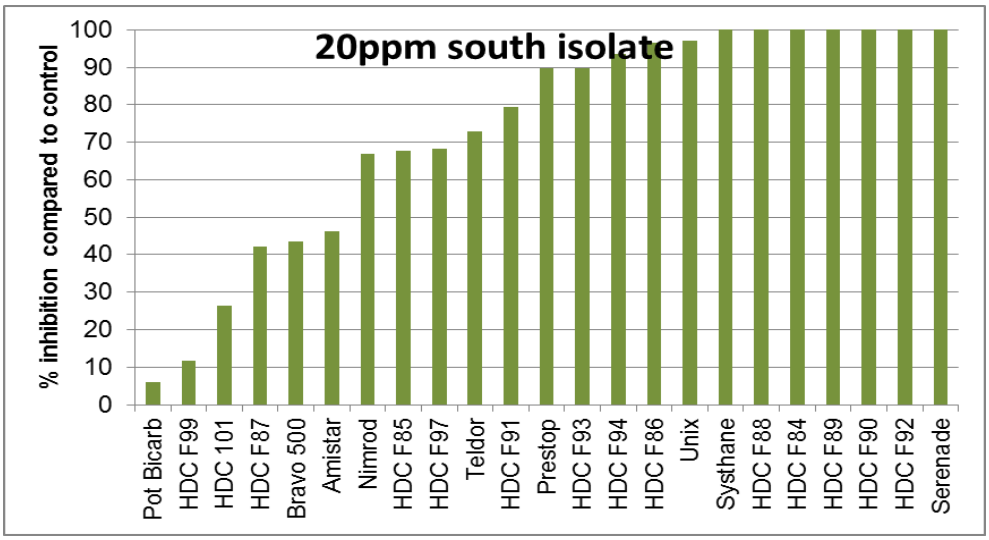
Serenade


20ppm south isolate
% inhibition compared to control
100
90
80
70
60
50
40
30
20
10
0
Pot Bicarb
HDC F99
HDC 101
HDC F87
Bravo 500
Amistar
Nimrod
HDC F85
HDC F97
Teldor
HDC F91
Prestop
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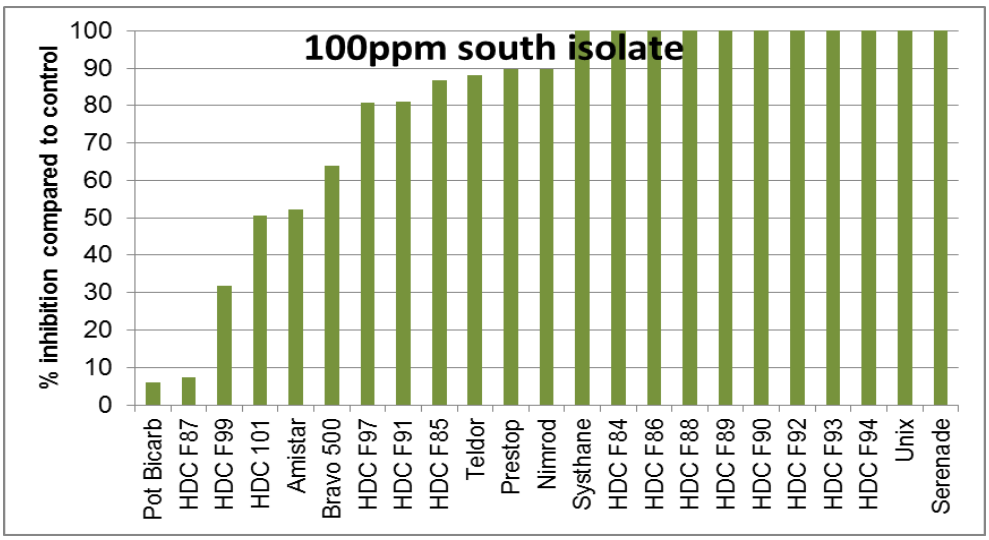
100ppm south isolate
% inhibition compared to control
100
90
80
70
60
50
40
30
20
10
0
Pot Bicarb
HDC F87
HDC F99
HDC 101
Amistar
Bravo 500
HDC F97
HDC F91
HDC F85
Teldor
Prestop
Nimrod
Systhane
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Unix
Serenade