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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PH0415
2. Project title: Developments of alternatives to the use of methyl bromide fumigation for in-transit treatments for alien invertebrate pests
3. Contractor organisation(s): The Food and Environment Research Agency
   Sand Hutton
   York
   YO41 1LZ
4. Total Defra project costs (agreed fixed price): £ 117,808
5. Project: start date ..... 01 April 2006
   end date ..... 31 October 2008

6. It is Defra's intention to publish this form.
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(a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.
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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

1) Various non-indigenous invertebrate pests including *Bemisia tabaci*, *Liriomyzia huidobrensis*, *Leptinotarsa decemlineata* and certain thrips and nematode species remain serious threats to the UK agricultural and horticultural industry. Many of these species are of serious economic concern as they are potentially very damaging to crops, difficult to control and many are also effective vectors of damaging plant viruses.

2) Currently the majority of quarantine based fumigations rely on methyl bromide. However, because of the phase-out of this fumigant in January 2005 alternative treatments are needed, though quarantine use of the gas is still permitted. A number of potential alternative treatments that may be suitable for quarantine disinfestation have been identified, including other fumigants, essential oils, ultra violet radiation and entomopathogenic fungi and nematodes.

3) The objectives of this study were to investigate the effects of these alternative treatments on both the pests and the plants on which they are likely to be treated. The combination of phytotoxicity and efficacy should provide an indication of the potential for the treatment to be utilised as a quarantine treatment.

4) Fumigating plants with Sulphuryl fluoride and Carbonyl sulphide caused severe plant damage and are therefore deemed off no use as alternative fumigants. Phosphine fumigations at 2g/m3 caused no plant damage after 24 hours exposure on Poinsettia and Impatiens and after 8 hrs exposure on Pelargoniums. No plant foliage damage was caused by the essential oils Fennel, Aniseed and Safrole.

5) Phosphine was an effective treatment against *Frankliniella occidentalis* and *Leptinotarsa decemlineata* but had no impact on nematode cysts. Total mortality of test insects was recorded well within the timescale where plant damage would potentially be a problem. This product has high potential for use as a quarantine treatment.

6) The essential oils were less efficacious and, at the temperatures achievable within the study, air saturation with the products did not provide a suitable level of efficacy against any of the pest species tested with the possible exception of Fennel against *Frankliniella occidentalis*. It is possible however that at higher temperatures greater levels of efficacy would be achievable (see Kostyukovsky et al, (2002) for some examples where this was the case).

7) Exposure of plant material to UV-C can produce phytotoxic effects such as scorching and some discolouration, however in this study the plants were treated when young and they soon grew through the

symptoms and the treated plants would have remained marketable following up to 1 minutes exposure. After five minutes however, the damage is likely to have been too visible to be acceptable to buyers even though, in most cases, the final, fully grown plant looked virtually indistinguishable from an untreated plant.

8) Exposing *Frankliniella occidentalis* adults, larvae and eggs to UV-C radiation had minimal impact on mortality. Thrips eggs exposed to treatments of 5 minutes in duration still hatched and produced viable offspring. UV-C light radiation has shown much potential to reduce *Liriomyzia huidobrensis* (South American leafminer) populations and an impact on fly emergence was noted after only 15 seconds exposure. Complete mortality of the fly was recorded after 7 minutes exposure. UV-C certainly has the potential for use against *L. huidobrensis* pupae and warrants further consideration.

9) Within the European Community the UK has 'Protected Zone' status to prevent spread of *Bemisia tabaci* (whitefly). Within this zone the primary concern is that adult whitefly imported on ornamentals such as poinsettia can infect tomatoes with tomato yellow leaf curl virus (TYLCV) and cucumbers with curcubit yellow stunting disorder virus (CYSDV) and cucumber vein yellowing virus (CVYV). These viruses are not currently present in the protected zone but have been detected in *B. tabaci* intercepted in the UK. Statutory action is taken to prevent live stages of the pest entering the UK. However, where outbreaks do occur measures must be taken to eradicate the population.

9) Entomopathogenic nematode biocontrol agents offer potential to control *B. tabaci*. Much work has investigated the efficacy of the nematode *Steinernema feltiae* against *B. tabaci*. This nematode has a high efficacy against second instar larval stages of *B. tabaci*. Also *S. feltiae* can be successfully integrated with several chemical insecticides commonly used in the UK for controlling *B. tabaci*. The nematode *S. carpocapsae* has also been cited as potentially having a higher efficacy against invertebrate pests, but this requires independent testing.

10) In order to support control and eradication approaches, further information is needed on potential biocontrol agents and their efficacy with a wider range of insecticides for the control of *B. tabaci*.

11) Direct suspension of the nematode (*S. carpocapsae*) in recommended label dose rates of the chemicals Calypso (thiacloprid), Chess (pymetrozine) and Admire (imidacloprid) have shown potential of proving viable options for IPM development. All three produced nematode infectivity levels above commercially viable requirements. Direct suspension with Oberon (spiromesifen) did not produce commercially acceptable nematode infectivity levels.

12) In the presence of insecticide residues good mortality of second instar *B. tabaci* was achieved after application of the nematode. In certain instances, for example, with thiacloprid and imidicloprid a higher mortality of *B. tabaci* larvae was obtained following sequential application of the chemical and nematode (86 and 71% respectively) compared to application of chemical alone (75 and 62% respectively).

13) *Steinernema carpocapsae* has proved itself to be of much potential use in the development of control measures against *B. tabaci*. This work has proved its high efficacy against second instar larval stages following spray application and has shown it to have a good compatibility with a wide range of chemical products and in some cases produce an added affect when applied sequentially.

14) It is not expected that there is one treatment that will have the same level of efficacy and broad spectrum applicability as Methyl Bromide and this work was developed in order to investigate some potential treatments and investigate the applicability of the efficacious treatments across a range of plant/pest combinations with a goal of drawing up treatment schedules for those treatments that are practicable. However, a cut in Defra funding meant that this project was terminated at the end of the second year, so these final outcomes were not achievable.

15) *Bemisia tabaci* remains one of the highest non-indigenous threats to UK horticulture and the incorporation of biopesticides, such as the nematode suspensions, into a broader IPM strategy should be investigated.

16) Discussions with the PHSI and Plant Health Policy representatives should be initiated to review the situations in which phosphine fumigation could be used. Consideration should then be given to the cost and practical implications of phosphine fumigation under those circumstances and where there is an economic case for undertaking the treatments, further investigations of the processes should be considered and treatment schedules developed.

17) The use of UV-C has shown some potential and could be investigated further, potentially within broader IPM systems. Consideration should be given, however, to how far away from having a method of treatment on a larger scale the industry is and what processes would need to be undertaken to get to a

point where larger scale treatment with UV-C is possible.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Introduction

The introduction of non-indigenious pests and diseases into a country can impact on both forestry and horticultural industries (McDonald et al, 1999), where crop damage and even complete loss may occur. Environmental damage has also been recorded following establishment of non-indigenous organisms (Murchie et al, 2003). New pests and diseases, many of which show high levels of pesticide resistance, will further increase pesticide use (Cahill et al, 1996). This has resulted in the need for alternative means of pest control to be devised (Cuthbertson et al, 2003a, 2003b, 2005a, 2005b, 2008). The European Union (EU) identifies a range of organisms of plant health importance whose introduction and movement around the EU are prohibited (Cheek & Cannon, 2002). Therefore, effective disinfestation treatments are vital if traded plants and plant products are to meet these legislatory requirements.

The majority of existing quarantine schedules rely on methyl bromide fumigation (Walters, 2001). Methyl bromide is a critical element in the pre-plant management of soil-borne pests and pathogens in high value fruit, nursery and ornamental crops, and in post-harvest management of pests on fresh produce and durable commodities (Schneider et al, 2003). Since the early 1990's, this fumigant has been known to break down under the influence of strong UV rays, and thus release bromide atoms which deplete the ozone layer (WMO, 1995). Increased environmental awareness of the effect of chemicals on the environment (Horowitz et al, 2003) and the phase-out of methyl bromide in industrialised countries in January 2005 has lead to a greater demand for alternatives to methyl bromide for invertebrate pest control to be devised (Giannakou & Karpouzas, 2003, Slusarski & Pietr, 2003) even though quarantine use of methyl bromide is exempt from the ban where there is no viable alternative. A number of potential alternative treatments that may be suitable for quarantine disinfestation have been identified. These include; heat treatments, composting, extreme controlled atmospheres and alternative fumigants (Walters, 2001).

The aim of the current project was to further investigate the use of alternative fumigants to methyl bromide as plant quarantine treatments and also to assess the effects of several natural plant volatiles as alternative fumigants to methyl bromide to provide complete mortality of various developmental life stages of the following invertebrate plant pests, *Liriomyza huidobrensis*, *Bemisia tabaci*, *Frankliniella occidentalis*, *Leptinotarsa decemlineata* and *Globodera rostochiensis*. The entomopathogenic nematode *Steinernema carpocapsae* was also screened both for its efficacy against *Bemisia tabaci* life-stages and for its compatibility with chemical insecticides.

Objectives

To take advantage of published and unpublished research into alternative treatment methods to the use of Methyl Bromide fumigation for intransit control of quarantine pests on imported plants and plant products (that under defined circumstances may also be applicable to clean up of outbreaks), by investigating and establishing the potential of selected methods in isolation and where appropriate in combination by:

1. Selecting target pests and host plant combinations: Liaising with a project Steering Group including Plant Health policy makers, Plant Health consultants and members of the Plant Health and Seeds Inspectorate to select priority invertebrate pests from different taxonomic groups for investigation. Select appropriate host plants on which they may be transported into the UK in trade for experimental work.
2. Reviewing the pest control methods for the selected pests: Selecting candidate control methods and summarising existing data on responses of different pest species, life stages etc to these methods, and on phytotoxic effects on the test plants.

3. Determine the effect of selected chemical treatments on plant foliage: Undertaking experimental work to determine phytotoxic effects of selected chemical treatments on chosen economically important plant commodities.

4. Filling treatment data gaps by experimentation for the selected treatments: Undertaking experimental work to fill gaps in datasets on the selected treatment methods/pest species.

5. Testing candidate treatments on mixed stage pest "populations": Reviewing findings and identifying the best treatments methods for each pest/life stage and considering the feasibility of combination treatments. Testing the candidate "best treatments" (including combination treatments) on mixed-stage pest populations infesting growing plants.

6. Considering potential for extension of pest treatment schedules to other plant hosts: If population tests are successful and extension is appropriate, investigating the potential for extending treatment to other plants selected by the Steering Group, or extending the project to other pest/treatment combinations if added hosts are not required.

7. Producing treatment schedules: If appropriate write practical treatment schedules for consideration by the PHSI.

8. Efficacy testing against requested quarantine species: The entomopathogenic nematode *Steinernema carpocapsae* will be screened for its efficacy against *Bemisia tabaci* instar life-stages and also for its compatibility with chemical insecticides. Results obtained will be compared against published data for *S. feltiae*.

Objective 1 Selecting target pests and host plant combinations.

A management structure for the project was agreed with the Defra project manager and consisted of a project Steering group (convened by the Defra project manager) with representatives of the major stakeholders (PHD, PHSI, Plant Health Consultants, Industry consultants) and the contractor's and Defra project managers. The Steering group met early in the project to discuss the organisms that should be investigated within the project. Choices were made on the basis of (i) risk, (ii) practicality of sourcing and handling insects (iii) lack of treatment options (iv) for the plants, the amount imported and likelihood that they will require treatment. The final decisions on the organisms to be used were as follow

Insect pests:
- Western flower thrips (*Frankliniella occidentalis*) to act as a model pest for *Thrips palmi*;
- Potato Cyst Nematode (*Globodera rostochiensis*) as a model for quarantine nematode species,
- Sweetpotato whitefly (*Bemisia tabaci*),
- Colorado beetle (*Leptinotarsa decemlineata*)
- South American leaf miner (*Liriomyza huidobrensis*).

Plant hosts:
- Pelargoniums
- Impatiens
- Poinsettias

Objective 2: Reviewing the pest control methods for the selected pests

Milestone 1: Complete review of control methods and prepare recommendations for those to be investigated under the project

A literature search covering published material and previous unpublished research undertaken by Fera in the field of alternatives to methyl bromide was completed and supplemented with information from other appropriate unpublished sources, largely gained through personal contacts of the science delivery group members with other national and international researchers in the field where appropriate. Discussions were held with the Project Steering Group on the potential approaches to take within this project and the following 6 fumigants were selected for further investigation:

Chemical fumigants which showed potential in previous work:
1) Sulphuryl fluoride; 2) Carbonyl sulphide; 3) Phosphine

Essential oils which had been shown to have some insecticidal properties in the literature (Kostyukovsky et al, 2002):
1) Fennel; 2) Safrole; 3) Aniseed

The potential of ultra violet light (as UV-C) was also investigated following interesting results in other areas of research within CSL (e.g. grain storage pests).

Objective 3. Determine the effect of selected chemical treatments on plant foliage:

Milestone 2: Complete work to fill gaps in published/unpublished datasets for the schedule for the first pest / treatment combination.

The first step in the investigation of these fumigants was to ascertain if there where any phytotoxic effects on the plant species under consideration.

Method:

Test fumigants
The fumigants investigated were sulphuryl fluoride, carbonyl sulphide, phosphine (as Frisen, a cylinderised formulation containing 2 % phosphine in nitrogen), and the essential oils of fennel, safrole and aniseed. The fumigant concentrations were measured using a Hewlett Packard 5890 series II gas chromatograph (GC) fitted with a flame photometric detector for phosphine and a thermal conductivity detector for sulphuryl fluoride. Fumigants were introduced into 1.7 m3 fumigation chambers at a concentration higher than the desired level. The concentration was measured and then reduced to the target concentration by partial evacuation of the chamber and the subsequent re-introduction of air.
The chambers were equipped with ports fitted with plastic sleeves that allowed test material to be introduced into the chambers and removed with minimal loss of concentration.

Test Plants
Rooted plants were obtained from a commercial source. During each fumigant trial an equal number were treated with the fumigant and an equal number stored at 3°C, 16:8 L:D as control. After the fumigation treatment cuttings were planted up and grown on for four weeks, during which visual inspections were made for plant foliage damage. Photographs were also taken as appropriate.

Fumigation times were as follows:
Sulphuryl fluoride – 1,2,4,8 hrs
Carbonyl sulphide - 8, 16, 24 & 72 hrs
Phosphine - 8, 16, 24 & 72 hrs
Fennel (*Foeniculum vulgare* essential oil) - 2, 4, 8 & 16 hours
Aniseed (*Pimpinella anisum* essential oil) - 2, 4, 8 & 16 hours
Safrole - 4, 8, 16, 24 & 48 hours.

Results:

Sulphuryl fluoride fumigations: all three plant species were severely damaged following exposure to this fumigant (20g/m3, 150C, 70% r.h). Even after only 1 and 2 hour exposures leaf burn was recorded and it was clear that even the shortest exposure led to damage that would make the plant unsaleable (Fig 1, Fig 2).

Figure 1: Poinsettia plants 27 hours post exposure to 20g/m3 Sulphuryl fluoride for 1 hour.

Figure 2: Pelargoniums 27 hours post exposure to 20g/m3 Sulphuryl fluoride for 2 hours.

Carbonyl sulphide fumigations: Within the first day post exposure, all plant species looked fine, however, following 2-3 days all plants showed signs of leaf burn and one week post exposure (150C, 70% r.h. 80g/m3) all plants were dead (Fig 3).

Figure 3: Poinsettia 1 week post exposure to 20g/m3 Carbonyl sulphide for 16 hours.

Phosphine fumigation: only after 72 hours exposure (150C, 70% r.h. 2g/m3) did poinsettia plants show any detrimental signs (leaves curled and crispy (Fig 4)). However, after one week plants from shorter time exposures were fine. The geraniums were more sensitive. One week following 72 hours exposure the plants were all dead. Impatiens after 16 hours exposure showed signs of leaf burn. Following a full weeks exposure to phosphine all plant species were killed.

Figure 4: Poinsettia 1 week post exposure to 2g/m3 Phosphine for 72 hours.

Fennel (*Foeniculum vulgare*), Aniseed (*Pimpinella anisum*) and Safrole fumigation: after all exposure times (up to either 16 or 48 hours in saturated air depending on the treatment) all three plant species were unharmed by exposure to these essential oils.

Table 1: Greatest number of hours exposure at which no signs of phytotoxicity were visible.

	Sulphuryl flouride	**Carbonyl sulphide**	**Phosphine**	**Fennel**	**Aniseed**	**Safrole**
Poinsettia	0	0	24	16	16	48
Impatiens	0	0	8	16	16	48
Pelargonium	0	0	24	16	16	48

Discussion
Discussion

From these investigations it is clear that however toxic to insects either Sulphuryl flouride or Carbonyl sulphide actually are, for these plant species at least, the damage to the plant is too great to allow their use. Hence, only phosphine and the essential oils showed any potential for further development as insect control agents as all the plants were able to tolerate exposure for a reasonable length of time without developing any obvious phytotoxic symptoms (Table 1). The other fumigants tested caused severe economic damage.

UV-C tests

Method:
A UVP CX-2000 crosslinker was used to generate the UVC at a wavelength of 254 nm. The crosslinker was calibrated using a UVP radiometer with UVX-25 sensor and found to deliver a light irradiance of approximately 9 mW/cm2. The test samples were placed approximately 9 cm from under the light source.

Small plants (ready for pricking out and with soil removed so roots fully exposed) were exposed to the UV-C light for the following time periods: 5 sec, 10sec, 30sec, 1min & 5min. There were 10 plants per exposure of each species. 10 plants of each species acted as controls, these were exposed to the same conditions only inside tinfoil wrapping to shield from the UV-C light. The controls underwent treatment for 5 mins.

A second trial involved flowers undergoing two exposures of UV-C light. The first exposure was followed 48hrs with a second exposure for the same period of time.

Following all treatments the plants were potted up and grown on within a glasshouse cubicle and observed for physical damage, with photographs taken. After two months the following parameters were measured for both treated and control plants: wet and dry weights and shoot height and root length.

Results

After two months in the glasshouse following the treatments there was not a noticeable difference in the measured parameters (Figs 5-7) suggesting that there is no long term effect on the development of the plants.

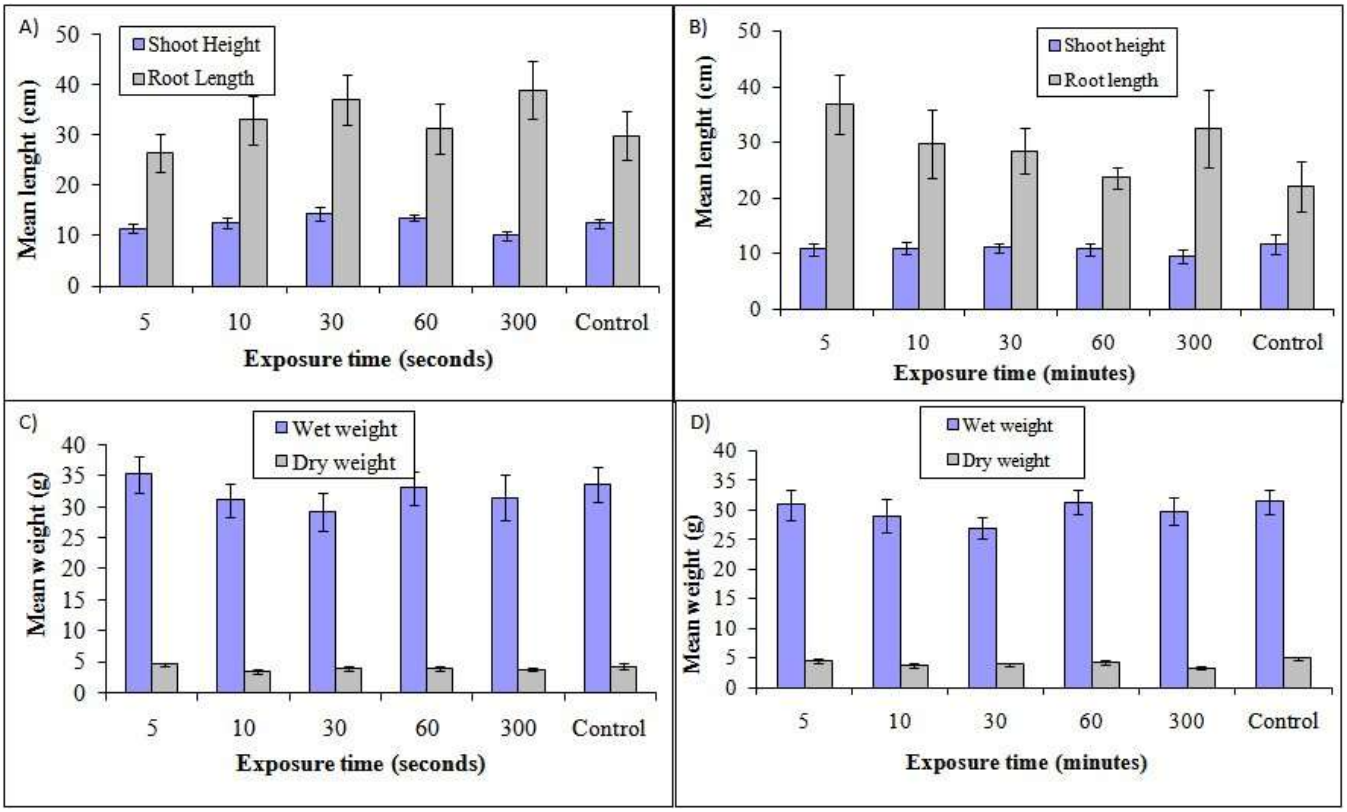

Figure 5: Measurements of shoot and root length (A & B) and the wet and dry weights (C & D) following exposure to UV-C once (A & C) or twice (with a gap of 48 hours (B & D)) of Pelargonium plants.

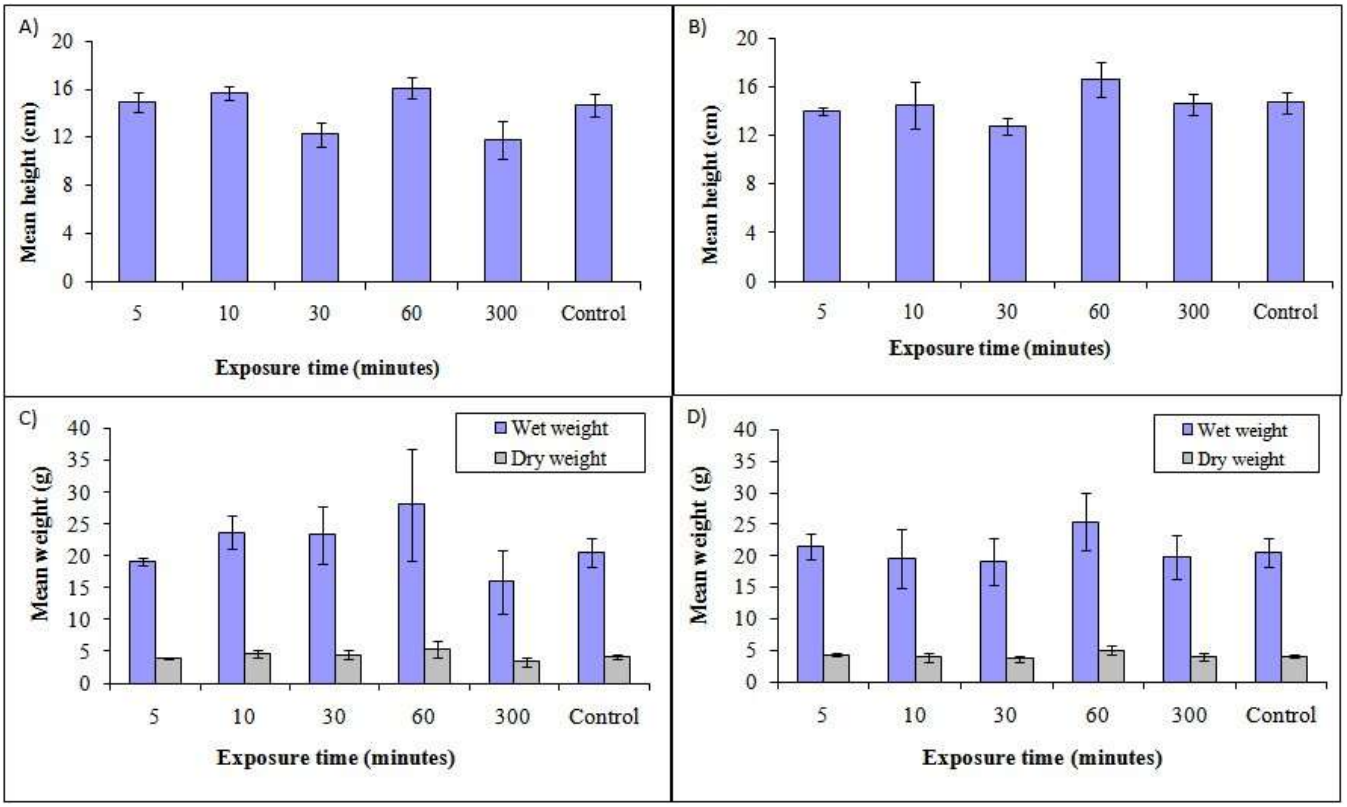

Figure 6: Measurements of shoot length (A & B) and the wet and dry weights (C & D) following exposure to UV-C once (A & C) or twice (with a gap of 48 hours (B & D)) of Poinsettia plants.

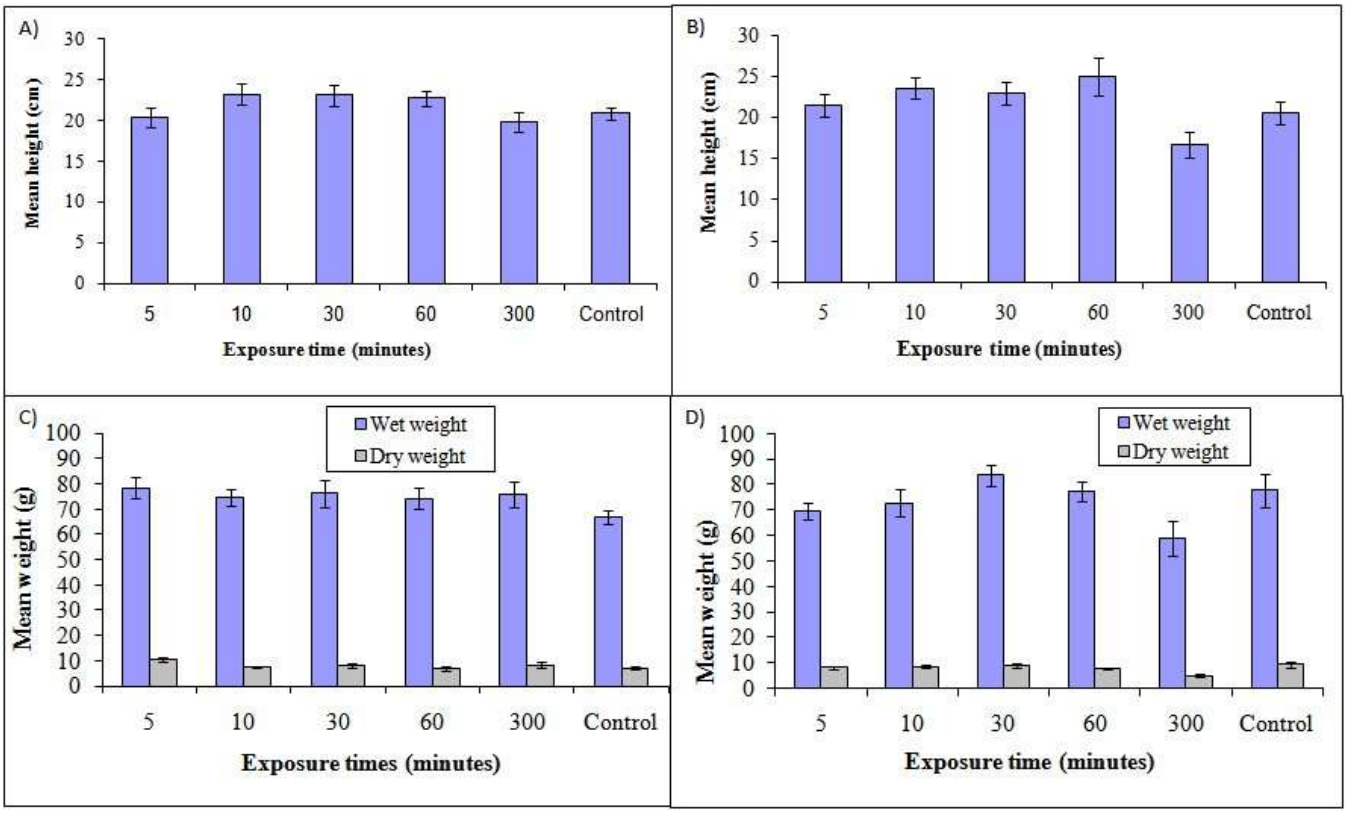

Figure 7: Measurements of shoot length (A & B) and the wet and dry weights (C & D) following exposure to UV-C once (A & C) or twice (with a gap of 48 hours (B & D)) of Impatiens plants.

Although the growth was not effected, there was some physical damage with scorching and some discolouration. The levels of this increased in all plant species tested the longer the plants were in the treatment. At the shorter time periods, the discolouration was restricted to the leaves exposed to the treatment and very little scorching was visible (figs 8-10).

Figure 8: Poinsettia plant 7 days after treatment of 5 minutes exposure to UV-C

Figure 9: Pelargonium plants 7 days after exposure to UV-C

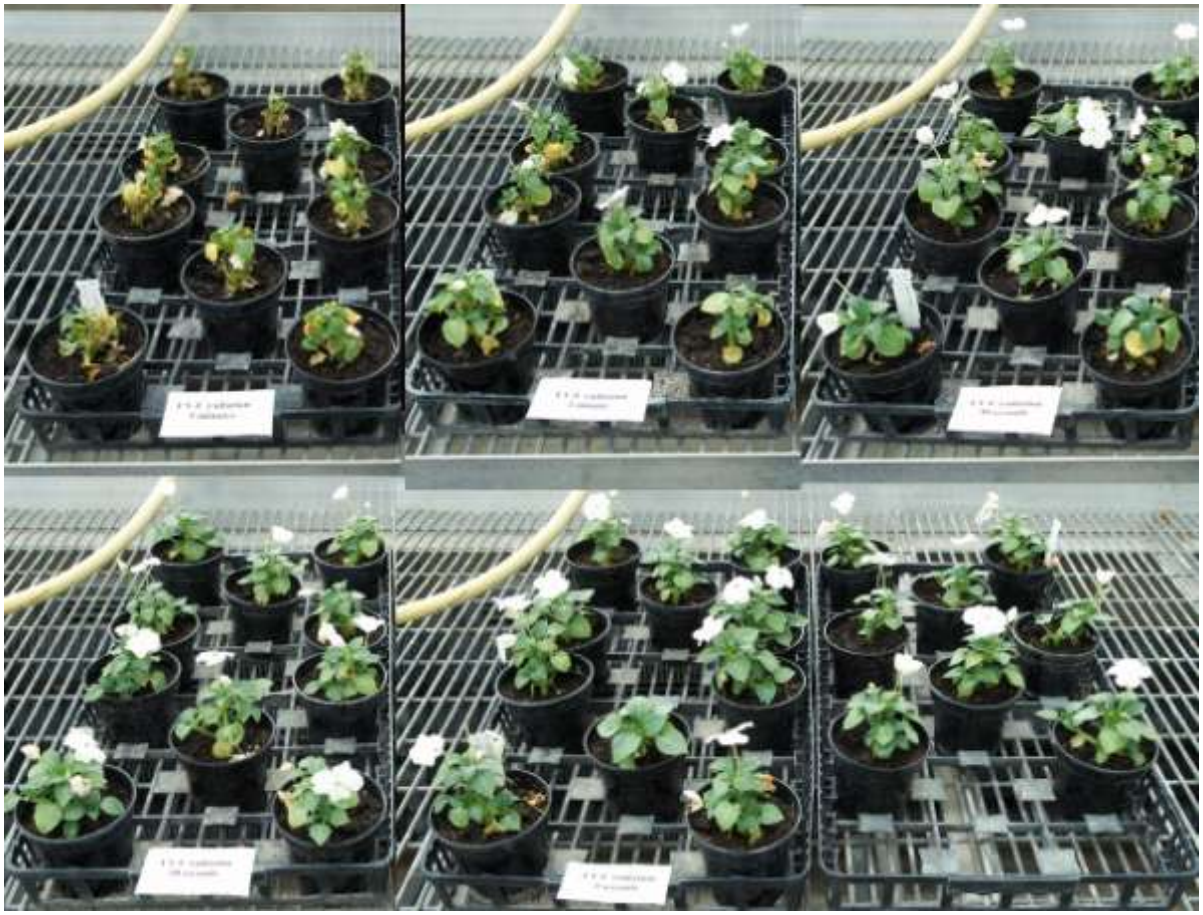

Figure 10: Impatiens plants 7 days after exposure to UV-C

Figure 11: Poinsettia plants 2 months post exposure to 5 minutes UV-C

Figure 12: Pelargonium plants 2 months post exposure to 5 minutes UV-C

Figure 13: Impatiens plants 2 months post exposure to 5 minutes UV-C

Table 2: Greatest number of seconds exposure to UV-C at which no signs of scorching were visible.

	Single Exposure	**Double Exposure**
Poinsettia	60	60
Impatiens	30	10
Pelargonium	60	30

Although there was some yellowing of the leaves, all the plants were able to withstand at least 30 seconds without scorching (Table 2) and after 2 months growth in the glasshouse following the a single exposure of 5 minutes, the plants are growing well with little sign of the effect of the treatments (Fig 11-13).

Discussion

These results show that there is definitely potential for UV-C treatments to be utilised as a pest control method as the level of phytotoxicity in the plants is relatively low at shorter treatment times and the plant is able to grow through them. Once the optimal timing for the delivery of a fatal dose to the pests has been determined, it will be necessary to look in more detail at the potential phytotoxicity effects at those levels as it is not entirely clear that the discolouration seen in some of the treatments is due to the treatment as some similar discolouration is occasionally also seen in the control.

Objective 4: Filling treatment data gaps by experimentation for the selected treatments

Method:

Test fumigants
The fumigants investigated were phosphine (as Frisen, a cylinderised formulation containing 2 % phosphine in nitrogen), and the essential oils of fennel, safrole and aniseed. The fumigant concentrations were measured using a Hewlett Packard 5890 series II gas chromatograph (GC) fitted with a flame photometric detector for phosphine and a thermal conductivity detector for sulphuryl fluoride. Fumigants were introduced into 1.7 m3 fumigation chambers at a concentration higher than the desired level. The concentration was measured and then reduced to the target concentration by partial evacuation of the chamber and the subsequent re-introduction of air. The chambers were equipped with ports fitted with plastic sleeves that allowed test material to be introduced into the chambers and removed with minimal loss of concentration.

Insect cultures
Stock cultures of *Bemisia tabaci*, *Liriomyza huidobrensis* and *Frankliniella occidentalis* and *Leptinotarsa decemlineata* were maintained following the methods of McDonald et al. (1999) under quarantine conditions within perspex cages as described for *B. tabaci* by Cuthbertson et al. (2003a,b). Nematodes were provided from stock cultures held by the Fera Nematology unit. Specimens were taken as required within ventilated containers containing fine green beans, as a source of food for the insects, for fumigation or UV-C light radiation. The nematodes were maintained within 'tea-sacs' (fine nylon bags) and placed within compost inside 3-inch flower pots. Sufficient specimens were maintained as controls.

Liriomyza huidobrensis – 15, 30, 45 seconds; 1, 3,5,7,10 mins (UV-C light radiation)
Frankliniella occidentalis – 1,2,4,6,8,16,24,72hrs (fumigations)
Leptinotarsa decemlineata – 1,2,4,8,24hrs (fumigations)
Globodera rostochiensis – 8,16,24,72hrs (fumigations)

Results

*W*estern flower thrips (*Frankliniella occidentalis*):

Phosphine:
Phosphine proved to be very effective at killing western flower thrips (WFT). After 4 hours exposure all thrips were recorded dead (Fig 14) and high mortalities were recorded with shorter exposure times, however, complete mortality was not achieved. WFT were then exposed to a range of doses of Phosphine for 4 hours to obtain a dose response curve (Fig 15). Complete mortality was obtained after 4 hours exposure to a concentration of 0.236 g/m3.

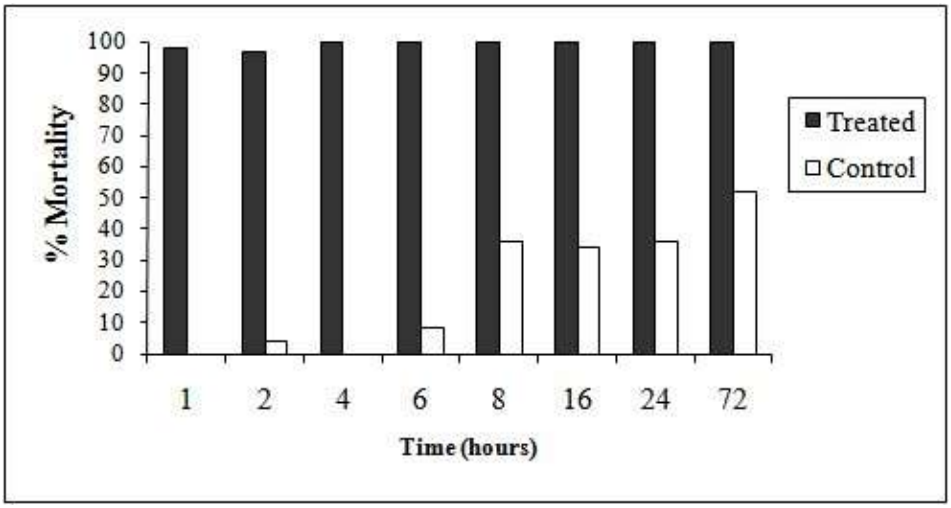

Figure 14: The effect of exposure of phosphine (2g/m3 at 15oC, 70% r.h.) on *Frankliniella occidentalis*

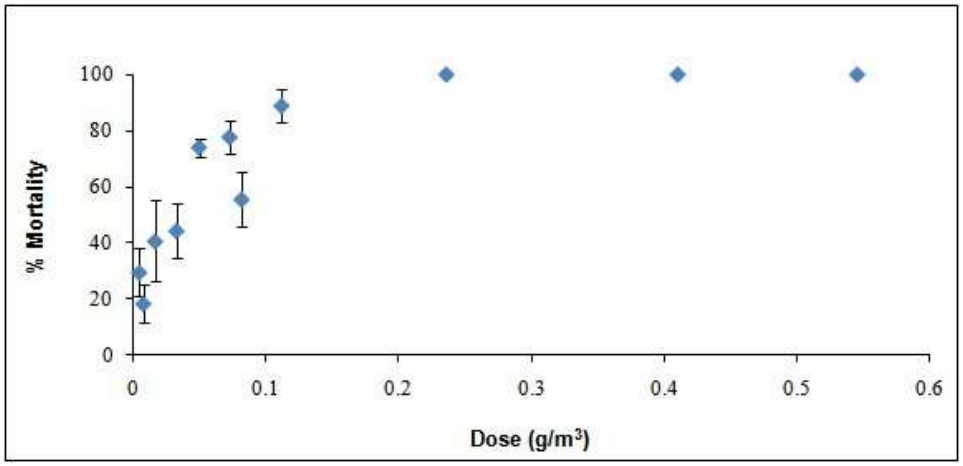

Figure 15: A dose response for exposure of *Frankliniella occidentalis* to phosphine (2g/m3 at 15 °C, 70% r.h.) following 4 hour exposures (mortality adjusted for control using Abbots formula).

Essential Oils:
The three essential oils showed variable results with Aniseed (Fig 16) and Safrole (Fig17) showing levels of mortality below 50%. The Fennel treatment showed a greater level of efficacy with over 60% mortality after 4 hours and 100% mortality after 24 hours (Fig 18).

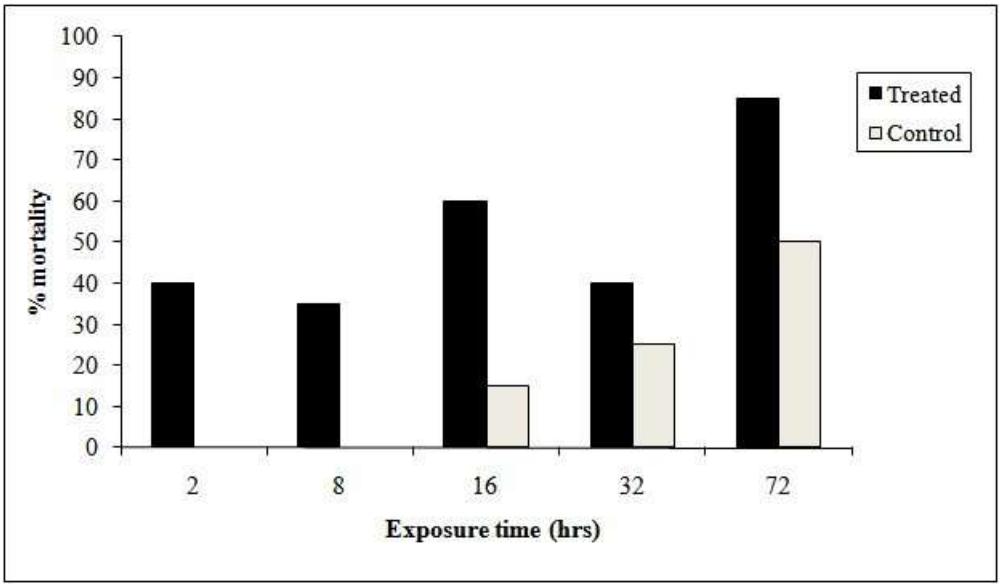

Figure 16: Effect of Aniseed (saturated air) on *Frankliniella occidentalis* at 20°C.

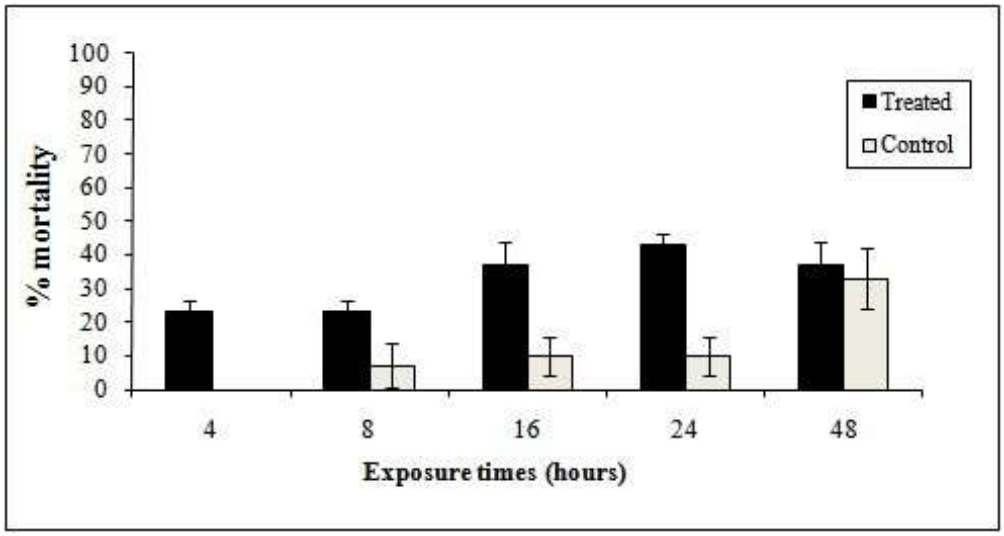

Figure 17: Effect of Safrole (saturated air) on *Frankliniella occidentalis* at 20°C.

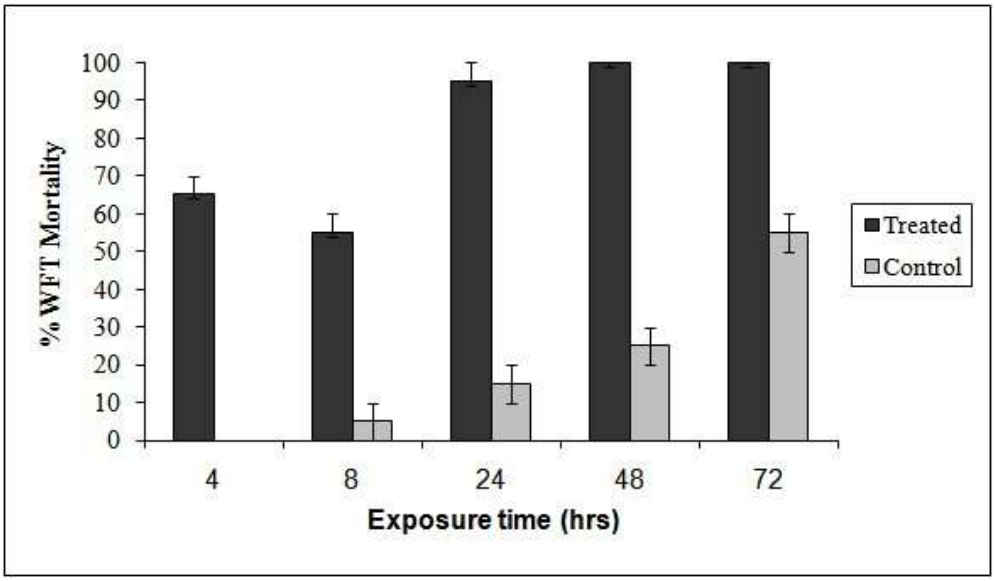

Figure 18: Effect of Fennel (saturated air) on *Frankliniella occidentalis* at 20°C.

Globodera rostochiensis

The most resistant life stage was chosen for the initial testing as if no suitable level of efficacy against the cyst could be found then the use of the method as a potential treatment will be significantly limited. All four chemicals were tested over 4 exposure times (up to 72 hrs) and in all of the tests, the treatments had no effect on the viability of the cysts, with less than 2.5% mortality in all the control and treated samples.

Colorado beetle (*Leptinotarsa decemlineata*)

This organism turned out to be very difficult to get hold of due to a change in the regulations surrounding the exporting of live insects from the US. A significant period of time was spent attempting to acquire them and eventually they were sourced from Europe and a courier was found who was prepared to ship live insects.

Following the first shipment, techniques were developed for the handling and culturing of the beetle (see Appendix 1: A Standard Operating Procedure (EFF/468 Working with *Leptinotarsa decemlineata* (Say), the Colorado potato beetle) and after a further delivery of eggs a population was maintained that was capable of providing a small number of insects for the experiments.

(A)  (B) 

Plate 1 (A) Colorado beetle (*Leptinotarsa decemlineata*) adult and (B) larvae.

Adult beetle fumigations

Adult beetles were exposed to Phosphine at a concentration of 2g/m3, 70% r.h., 20°C for a number of exposure periods. 2g/m3 is a high dose; the aim was to start high to see if this treatment has any potential. In gas tight situations a normal dose would be about 1g/m3 but in most storage situations a dose of upto 5 g/m3 would be undertaken in the expectation that a lot would be lost through leakage. All beetles were killed after a 2 hour exposure. Following this result, tests at a range of doses and timescales were undertaken to obtain a dose response curve for adult beetles (Fig 19). The following concentrations were tested: 2g/m3, 1g/m3, 0.5g/m3, 0.12g/m3. All beetles were killed after 1 hour exposure to 0.5g/m3 (and above). Following an exposure to 0.12g/m3 for 2 hours no mortality was recorded, however from 4hrs, 100% mortality was found.

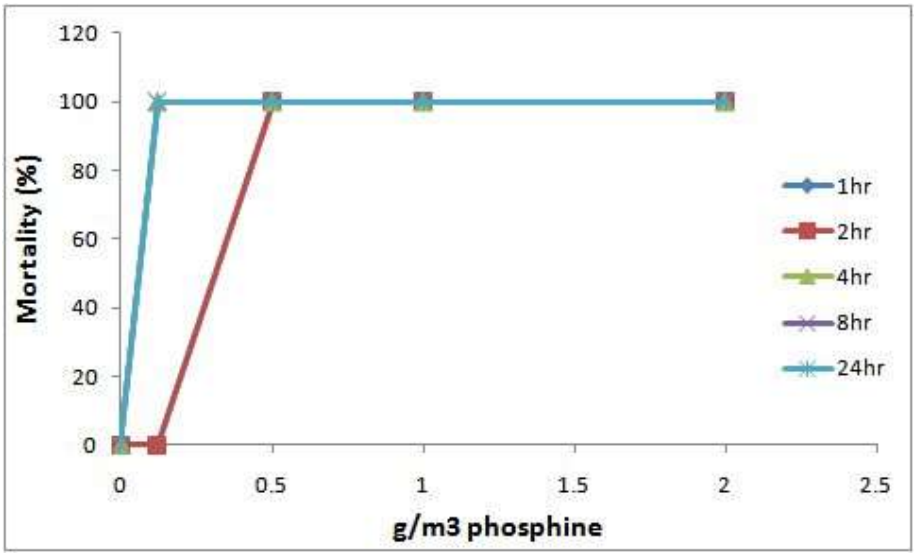

Figure 19: Dose response of Colorado Beetle adults to phosphine fumigation at 20°C

Egg fumigations:

Small scale unreplicated testing at 0.5, 1.0 and 2.0 g/m3 of phosphine suggested that at 0.5, 1.0 g/m3, 24 hours exposure can lead to egg mortality and 2 hrs exposure at 2.0 g/m3 will also lead to egg mortality.

Bemisia tabaci

The literature suggested that Safrole was an effective treatment against Thrips species as well as *Bemisia tabaci* (Kostyukovsky et al, 2002), so given the relatively poor results of the treatment against WFT it was decided to try and see if the results against *B. tabaci* were similar to that already published as this may give some insight into whether the treatment just happened to be poor against WFT or if it was possible that there was something wrong with the techniques used.

In these tests (Fig 20), the efficacy against *B. tabaci* was low, with barely 30% mortality reached after 48 hours exposure, much lower than the efficacy (100%) suggested in the literature (Kostyukovsky et al, 2002). However, we were unable to measure the exact dose the organisms were exposed to, so the comparison is not exact.

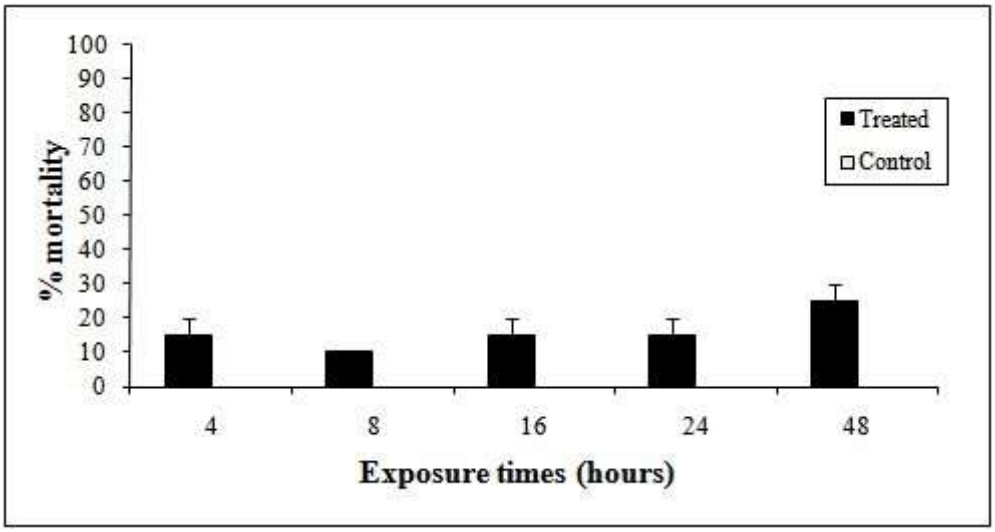

Figure 20: Effect of Safrole (saturated air) on *Bemisia tabaci* at 20°C.

Discussion

The data highlights that Phosphine has significant potential as a fumigant for quarantine purposes as it has a relatively low phytotoxicity and a relatively high efficacy against WFT and the colorado beetle. Although the essential oils showed no phytotoxicity, they had low efficacy as well in most cases, with the possible exception of Fennel against WFT. The differences in the efficacy found in these studies and that of Kostyukovsky et al (2002) could be explained by differences in technique and temperature, leading to the organisms being exposed to lower doses. None of the treatments had an effect on the nematode cysts.

UV-C tests on insects

Method

Ten *Liriomyza huidobrensis* pupae were placed into ventilated glass tubes. These then underwent the following UV-C light exposure times: 15, 30, 45 seconds; 1,3,5,7,10 minutes.

Green beans were placed in the *Frankliniella occidentalis* culture for 48hrs in order for eggs to be laid on them. The beans were then taken and placed in ventilated glass tubes. These then underwent the following exposure times: 0.5, 1, 5 minutes

Nematode cysts were also exposed for 0.5, 1, 5 minutes within tea-sacs.

Following exposure all insects were returned to their original CE cabinets and normal culturing resumed. The *L. huidobrensis* and *F. occidentalis* were both assessed for adult emergence and the nematode cysts for viability. The treated specimens were assessed against appropriate controls for each species.

Results

Western flower thrips (*Frankliniella occidentalis*):

Exposing WFT adults, larvae and eggs to UV-C light radiation had minimal impact on mortality. Only after 5 minute exposures was some morality recorded in regards to adult and larval lifestages. Thrips eggs exposed to treatments of 5 minutes in duration still hatched and produced viable offspring.

Nematode cysts:

UV-C light radiation had no impact upon nematode cysts. Further investigation is probably necessary to determine the efficacy of the light in penetrating soil material.

Leafminer pupae (*Liriomyzia huidobrensis*) :

UV-C light radiation has shown far greater potential to reduce *L. huidobrensis* population development. An impact on fly emergence was noted even after only 15 seconds exposure (Fig 11). Complete mortality of the fly was recorded after 7 minutes exposure.

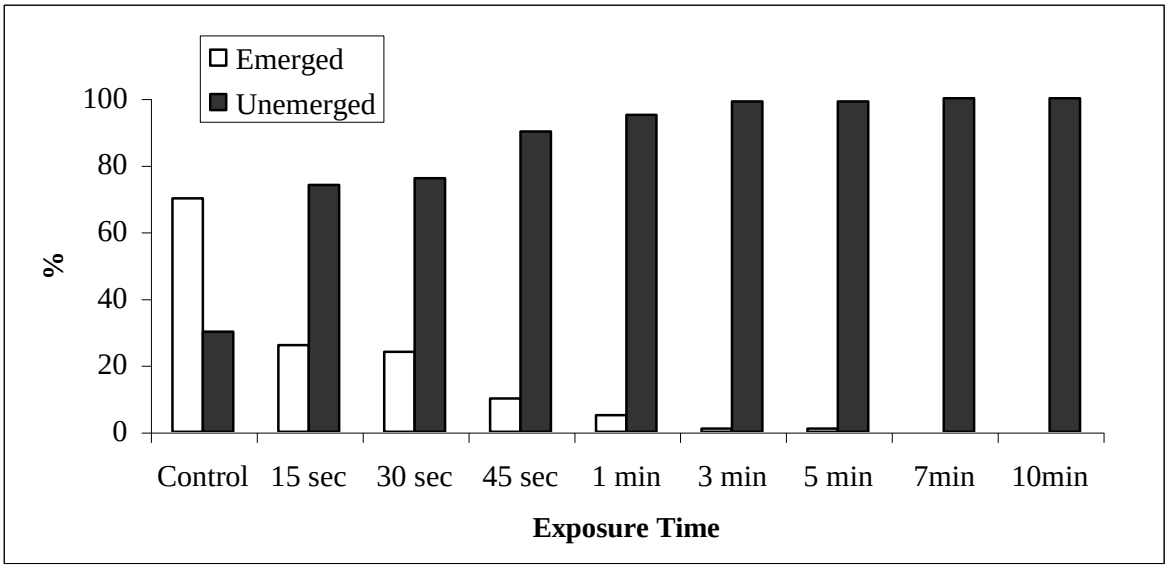

Fig 21. Impact of UV-C light radiation on emergence of *Liriomyza huidobrenis* pupae

Discussion

The utilisation of UV-C treatments has potential for treatment of certain pests, but it also has potential drawbacks e.g. phytotoxicity after relatively short timescales and the target organisms need to be directly exposed as the UV-C does not penetrate well through soil (for example). *Liriomyza huidobrensis* pupae appear to be highly susceptible to UV-C and are likely to be found on the leaf or on the soil so will be potentially in an exposed position where UV-C treatment will be most effective.

Objective 5: Testing candidate treatments on mixed stage pest "populations":

Objective 6: Considering potential for extension of pest treatment schedules to other plant hosts:

Objective 7. Producing treatment schedules:.

The three objectives above (5, 6 & 7) were scheduled for the final year of the project, however due to funding pressures within Defra, the final years work was cancelled, so these objectives have not been completed or reported on.

Objective 8: Efficacy testing against requested quarantine species

Efficacy of *Steinernema carpocapsae* against *Bemisia tabaci* immature stages

Nematodes were obtained as Capsanem (Koppert Biological Systems Ltd, UK). *Bemisia tabaci* were cultured following the methods of Cuthbertson et al. (2003a, 2005b) under quarantine conditions in Perspex cages on Poinsettia (*Euphorbia pulcherrima* c.v. Lilo Pink) at 23 ± 1°C and a 16:8-h Light:Dark (L:D) regime with an artificial dawn and dusk.

Following the methods of Cuthbertson et al. (2003a, 2005b) plants were divided into four groups (one for each instar stage with 12 plants in each group). For each group, four clip cages were positioned on individual leaves of each plant (*Verbena hybrida* cv. Quartz Scarlet). Two male and five female *B. tabaci* were added to each cage and incubated for 48 h at 25±1°C, 65% relative humidity (r.h.) and a 16:8-h L : D regime to allow egg laying. After this period the adults were removed and the leaves labelled. Each group of plants was incubated in a controlled environment cabinet under the above conditions for the required period of time (ranging from 1-3 weeks) to reach the appropriate instar. At 25°C it took approximately 7 days for first instar to develop through, 12 days for second instar, 15 days for third instar and finally 21 days for fourth instar development. This allowed all four stadia to be available for experimental use on the same treatment date.

Application of nematode:
All four stadia (first, second, third and fourth instar larvae) of *B. tabaci* were subjected to a nematode suspension (10,000 *S. carpocapsae* IJ ml-1 with 0.02% the non-ionic wetting agent Agral (Syngenta Crop Protection Ltd., UK; active ingredient: alkyl phenol ethylene oxide). Treatments were applied to run-off using a Hozelock Polyspray 20

hand held sprayer (Hozelock Ltd., Aylesbury, UK) with a cone nozzle. Treatments resulted in approximately 160 *S. carpocapsae* IJ per cm2 of leaf area. Control plants were subjected to either a water control or water and Agral mix (0.02%) solution. There were four nematode treated, four water and Agral mix treated, and four water treated control plants for each instar (12 plants in total). Following treatment, the host plants were placed back into an environmental chamber while still wet and maintained at 20 ± 1°C, 85% r.h. for 10 h and a 12:12-h D:L regime. After 72 h, counts of dead individuals (determined both by no larvae movement and the presence of nematodes in the larvae) per infested leaf (4 leaves per plant) were recorded. Analysis of Variance (ANOVA) was performed for the data in the open source statistical programme R. ANOVA, a general technique, is used to test hypothesis concerning means when there are several populations.

Direct compatibility of *Steinernema carpocapsae* with chemical insecticides

Following the method of Cuthbertson et al (2003b), infective juveniles (IJ) of *S. carpocapsae* were suspended (500 IJ/ml) in solutions of four insecticides commonly used for *B. tabaci* control in the UK. All the insecticides were diluted to the recommended rates for application to protected ornamentals in the UK: imidacloprid as Intercept 70 WG (0.2g/l water, a.i. 70% w/w, The Scotts Company Ltd, Ipswich, UK), spiromesifen as Oberon (0.5 ml/l water, a.i. 24% w/w, Bayer CropScience Ltd, Cambridge, UK), thiacloprid as Calypso (0.45 ml/l water, a.i. 48% w/w, Bayer CropScience Ltd, Cambridge, UK) and pymetrozine as Chess WG (0.2g/l water, a.i. 50% w/w, Syngenta Crop Protection Ltd, Cambridge, UK). Suspensions were prepared in 50 ml glass beakers, which were then sealed with Parafilm and incubated at 20 ± 1°C in the dark for 24 hours.

The infectivity of the nematodes was then assessed using the method of Fan and Hominick (1991) sand washed, autoclaved and dried before 4% water by volume was added. This was then used to fill 30 cm3 universal tubes. One ml of nematode suspension (diluted to contain 120 IJ/ml) was introduced into a 4 cm deep hole in the sand that was then filled by shaking the tube. A single *Galleria mellonella* L. (Lepidoptera: Pyralidae) larva was added to the surface of the sand before the tube was sealed, inverted and incubated at 20°C for 3 days. After this period the larva was removed from the tube, washed in water and maintained on moistened filter paper in a Petri dish for a further 24 hours. The larvae were then dissected. The number of nematodes present were counted and expressed as a percentage of those originally introduced into the tubes. The procedure was replicated 9 times for each of the four active ingredients. A control of nematode suspension in water was maintained and assessed in the same manner. The results were analysed by generalised linear model with logit link function (Genstat 9 statistical package). Post-hoc pairwise comparisons were performed using the standard errors of the differences based on the residual deviance.

Compatibility of *Steinernema carpocapsae* in the presence of insecticide residues

Infestation of plants
Following the protocol of Cuthbertson et al. (2003b) 30 tomato plants (*Lycopersicon esculentum* c.v. Moneymaker) at the ten leaf stage were exposed to adult *B. tabaci* for egg laying. Five clip cages, modelled on those described by MacGillivray and Anderson (1957), each containing two male and five females were placed on each plant (one cage per individual leaf) and incubated for 48 hours at 25 ± 1ºC, 65% relative humidity (r.h.) and 16:8-h L:D to allow egg laying, after which adults were removed. The plants were then incubated for a further 12 days to allow eggs to hatch and larvae to reach the second stadium (Butler et al.,1983; Bethke, 1991; Wang, and Tsai, 1996), the stage most susceptible to *S. carpocapsae* infection. The plants were then divided into four treatment groups (see Table 3) for application of insecticides.

Treatments
A combination of two sequential treatments as defined in the legend of Table 3 was applied to each plant. The first coincided with the emergence of second instar *B. tabaci* and consisted of either an insecticide applied at the recommended dose rate for application to ornamentals in the UK or a water control. Three treatment groups received foliar applications of insecticides (spiromesifen, thiacloprid and pymetrozine). The plants were sprayed to run-off using a Hozelock® Polyspray 2 hand held sprayer with a cone nozzle (Hozelock Ltd, Aylesbury, UK). The leaves were allowed to dry before being returned to the conditions defined for infestation with *B. tabaci*. To allow time for the systemic insecticide (imidacloprid) applied to the fourth group to be taken up by the plants, 30 ml diluted product was added to the soil three days (Cuthbertson et al., 2003b, 2005a) before the treatment date for foliar applications. Control plants received the equivalent volume of water (30 ml).

The second treatment was applied 24 hours later and consisted of either a suspension of 10,000 *S. carpocapsae* IJ/ml with 0.02% of the non-ionic wetting agent Agral (Syngenta Crop Protection Ltd., Cambridge, UK; active ingredient: alkyl phenol ethylene oxide) or a water control. Treatments were again sprayed to run off following the methods of Cuthbertson et al. (2003b). There were approximately 160 *S. carpocapsae* per cm2 of leaf surface. Following the second treatment, the plants were returned to the environmental chamber while still wet and maintained at 20 ± 1ºC, 85% r.h. and 12:12-h D:L period (treatments were co-ordinated so that plants were treated at the start of the dark period).

Assessment of efficacy
The numbers of live and dead larvae (determined by no movement and/or the presence of nematodes in the larvae were recorded per infested leaf (4 leaves per plant) 72 h after the second treatment was applied for each replicate. The proportion of larvae dead after 72 h was analysed by generalised linear model with logit link function (Genstat 9 statistical package). Post-hoc pairwise comparisons were performed using the standard errors of the differences based on the residual deviance.

Second and third instar larvae proved most susceptible to nematode infection (Fig 22). This was similar to what was established for *S. feltiae* by Cuthbertson et al, (2003a) previously in Plant Health funded project PH0157.

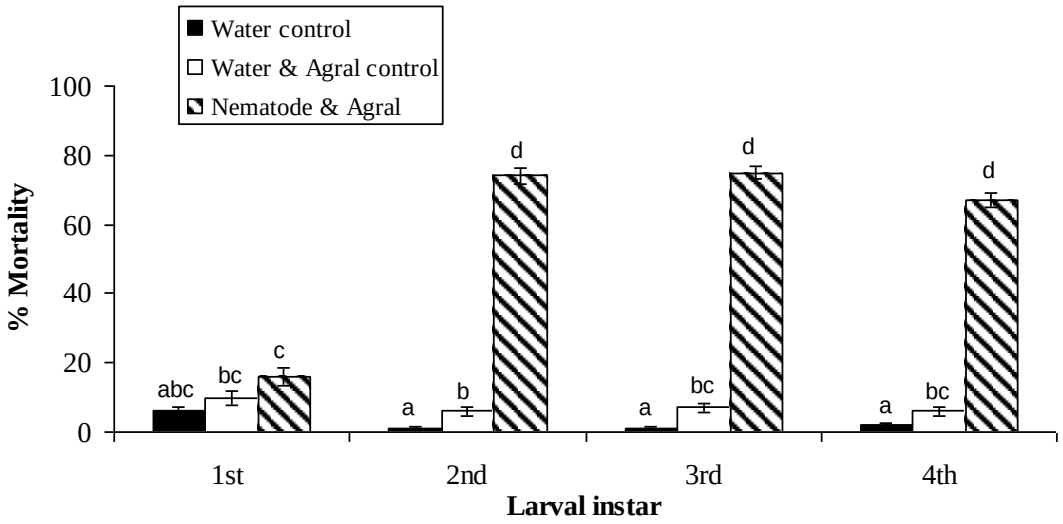

Figure 22. The susceptibility of the immature stages of *Bemisia tabaci* to the entomopathogenic nematode *Steinernema carpocapsae* (10,000IJ/ml) on verbena plants at 20 ± 1°C, 85% r.h. and 12:12 D:L. Bars are standard error (±SEM) of the mean.

This work has since been published in the international journal, *Russian Journal of Nematology* (2007) 15: 153-156. The abstract of the paper is as follows:

"The efficacy of the entompathogenic nematode *Steinernema carpocapsae* (Wesier) against the larval stages of the sweetpotato whitefly *Bemisia tabaci* Gennadius on verbena (*Verbena hybrida* c.v. Quartz Scarlet) foliage was investigated. Both second and third instar proved most susceptible to nematode infection. The potential of *S. carpocapsae* to be incorporated into integrated pest management strategies for the control of *B. tabaci* is discussed".

A copy of this published paper can be provided upon request.

Direct compatibility of *Steinernema carpocapsae* with chemical insecticides

Following the methods of Cuthbertson et al. (2003b) developed previously in PLH funded PH0157, impact of both direct suspension and chemical residues on nematode efficacy was investigated. Direct suspension of *S. carpocapsae* with the chemicals Calypso (thiacloprid), Chess (pymetrozine) and Admire (imidacloprid) have shown potential of proving viable options for IPM development (Figure 23). All three produced nematode infectivity levels above commercially viable requirements (>15%). Direct suspension with Oberon (spiromesifen) did not produce commercially acceptable nematode infectivity levels.

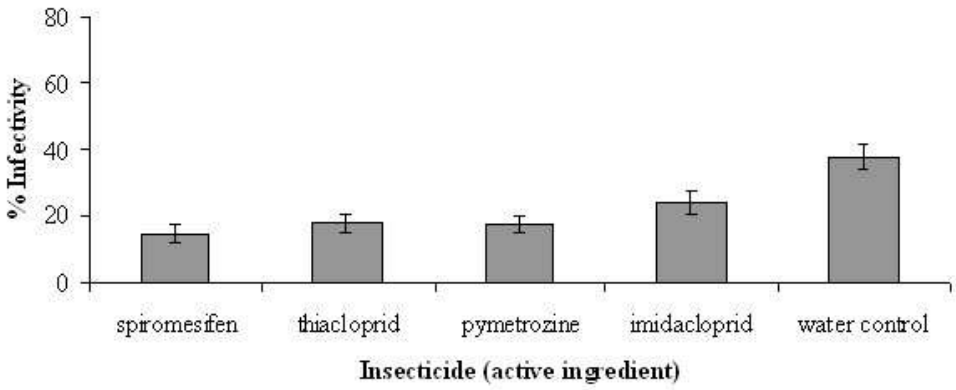

Figure 23. *Steinernema carpocapsae* infectivity (mean ± SEM) against *Galleria mellonella* larvae exposed to 120 infective juveniles (IJ) measured 72 h after a 24 h exposure of IJ to chemical insecticides or water control.

Compatibility of *Steinernema carpocapsae* in the presence of insecticide residues

In the presence of insecticide residues good mortality of second instar *B. tabaci* was achieved (Table 3). In certain instances, for example with thiacloprid and imidicloprid, a higher mortality of *B. tabcai* larvae was obtained following sequential application of the chemical and nematode (86 and 71% respectively) compared to only 75 and 62% respectively following application of chemical alone.

Table 3. The effect of chemical residues on verbena plants on the infectivity of *Steinernema carpocapsae* (ca 160 IJ per cm²) against second instar *Bemisa tabaci* larvae. Data represent % mortality.

1st appl.	Water	Chemical	Water	Chemical
2nd appl.	Water	Water	Nematode	Nematode
Spiromesifen	0	96	75	94
Thiocloprid	0	75	75	86
Imidicloprid	0	62	75	71
Pymetrizine	0	71	75	63

This work investigating the integration of *S. carpocapsae* with chemical insecticides has also since been published in the international journal *Insect Science* (2008) 15: 447-453. The abstract of the paper is as follows:

"The integration of chemical insecticides and infective juveniles of the entomopathogenic nematode *Steinernema carpocapsae* (Wesier) (Nematoda: Steinernematidae), to control second instars of the sweetpotato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) was investigated. Using a sand bioassay, the effects of direct exposure of *S. carpocapsae* for 24h to field rate dilutions of four insecticides (spiromesifen, thiacloprid, imidacloprid and pymetrozine) on infectivity to *Galleria mellonella* larvae were tested. Though all chemicals tested, except spiromesifen, produced acceptable nematode infectivity rates, they were all significantly less than the water control. The effect of insecticide treatment (dry residues of spiromesifen, thiacloprid and pymetrozine and soil drench of imidacloprid) on the efficacy of the nematode against *B. tabaci* was also investigated. Nematodes in combination with thiacloprid and spiromesifen gave higher *B. tabaci* mortality (86.5 and 94.3% respectively) compared to using nematodes alone (75.2%) on tomato plants. There was no significant difference in *B. tabaci* mortality when using the chemicals imidacloprid, pymetrozine and spiromesifen in conjunction with nematodes compared to using the chemicals alone. However, using thiacloprid in combination with the nematodes produced significantly higher *B. tabaci* mortality than using the chemical alone. The integration of *S. carpocapsae* and these chemical agents into current integrated pest management programmes for the control of *B. tabaci* is discussed."

A copy of this published paper can also be provided upon request.

Discussion

Fumigation is still one of the most effective methods for the protection of stored food, feed stuffs and other agricultural commodities from insect infestation (Kostyukovsky et al, 2002). The two main fumigants that have been in use are methyl bromide and phosphine. However, methyl bromide has been identified as a main contributor to ozone depletion (WMO, 1995), and has now been phased out in developed countries except for quarantine and pre-shipment and for uses granted a critical use exemption. As for phosphine, there are increasing reports of chemical resistance of pests to this fumigant (Nakakita & Winks, 1981; Tyler et al, 1983). The replacement of methyl bromide, classed as one of the most efficient soil fumigants for the last 50 years (Giannakou & Karpouzas, 2003) with alternative chemical treatments poses a major problem for the treatment of many insect pest species and also quarantine insects in transit. The development of potential non-methyl bromide quarantine treatments will allow horticultural growers to eliminate the use of an environmentally damaging chemical while still protecting the industry from losses that could be caused by the introduction of new pests and diseases.

The general levels of pest control recorded in this study following exposure to phosphine were promising. The phosphine treatments also did not appear to be damaging to the host plants and only treatments of more than 16 hours exposure proved detrimental to some of the plant foliage investigated. In addition, it may be feasible to use this treatment with other plant species that are more tolerant of the fumigant. The other gas fumigants tested (Sulphuryl fluoride and Carbonyl sulphide) caused severe damage to plant material and therefore are not commercially useful. In regards to insect control, treatments with phosphine at the lower temperature of 15°C resulted in complete mortality of all *Frankliniella occidentalis* adults after a 4 hour exposure. It also proved successful in killing Colorado beetles after only 2 hours exposure at a dose of 0.5g/m3.

The essential oils investigated had no impact against the nematodes and little effect upon the insect pest populations in the current study. However, they could be more effective at higher temperatures (i.e. greater doses achievable) as Kostyukovsky et al (2002) showed. Practically, it may be that the temperatures likely to provide better efficacy (i.e. 30°C) will not be attainable at the location where the treatment will need to take place.

Phosphine would appear to have the most potential for development as a quarantine fumigant out of the chemicals tested where the treatment temperature and duration both need to be kept to a minimum to limit plant damage whilst high levels of pest mortality are obtained.

The Ultraviolet (UV) portion of the light spectrum is widely used as a germicide and as an attractant for insects (Bruce, 1975) and for the surface disinfection of insect eggs (Guerra et al, 1968). Numerous studies have considered the possibility of using UV-rays to control, or at least to suppress development of various species of stored product insects (Faruki, 2005; Faruki et al, 2005). In the current study only *L. huidobrensis* pupae proved highly susceptible to UV-C radiation. A significant reduction in pupae emergence was observed following exposure to UV-C, even after as little as 15 seconds. *Liriomyza huidobrensis* pupae are likely to be found on the leaf or on the soil so will be potentially in an exposed position where UV-C treatment will be most effective. The other pest species' proved less susceptible, especially the nematode cysts contained with soil, where no impact was recorded.

On testing against plant material, some initial burning of foliage was noted, however, when the plants were kept under optimal growing conditions, they recovered and produced new foliage growth. They went on to flower and bloom as normal. At this stage no noticeable difference from control plants could be recorded. UV irradiation may well prove a very safe and clean method for disinfestations of plant products in transit, however, more comprehensive research is necessary to understand the plant/pest combinations where this technique would be appropriate and eventually what the mechanisms for treatment will be. It should be noted that appropriate H&S considerations should be made as "UVC is absorbed in the outer dead layers of the epidermis. Accidental overexposure to UVC can cause corneal burns, commonly termed welders' flash, and snow blindness, a severe sunburn to the face." (Health Physics Society, 2009).

Successful IPM strategies not only rely upon knowledge of the biology and feeding behaviour of a given control agent but also on the most susceptible stage of the pest to target. This study has demonstrated that *S. carpocapsae* can significantly reduce *B. tabaci* populations on verbena foliage with the second and third instar stages proving most susceptible to infection. This result is similar to that obtained for *S. feltiae* on tomato and slightly higher for that recorded on verbena foliage (Cuthbertson et al, 2003a) and for the fungus *Lecanicillium muscarium* (Petch) against *B. tabaci* on tomato and verbena foliage (Cuthbertson et al, 2005b) (PLH funded project: PH0157). Thus, *S. carpocapsae* offers the potential for development as a third biological agent in the IPM strategy for *B. tabaci*.

An extensive range of insecticide and nematode combinations have been reported for control of various invertebrate pest species. Within the UK only Cuthbertson et al. (2003b) (PLH funded project: PH0157) has investigated the combination of chemicals routinely used for the control of whitefly with the nematode *S. feltiae*. In order to improve the efficacy of an IPM system, other biocontrol agents must be screened for their suitability for

inclusion. This study therefore also investigated the incorporation *of S. carpocapsae* and chemicals into the IPM strategy for control of *B. tabaci*.

Insecticide resistance is an ever increasing problem within invertebrate pest control. Many commonly used insecticides, for example buprofezin and imidacloprid have now become ineffective in the UK against *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae) (glasshouse whitefly) by the widespread appearance of resistant populations. *Bemisia tabaci* have also been shown to have a degree of resistance to imidacloprid, adding to the urgency of the development of alternative IPM strategies.

The chemical groups most toxic to *Steinernema* spp. and *Heterorhabditis* spp. (Rhabditida: Heterorhabditidae) are thought to be organophosphates and carbamates. Studies investigating direct exposure of entomopathogenic nematodes to diluted pesticides have shown that nematode viability and virulence can be reduced (Zimmerman & Cranshaw, 1990). However, effective field control of lepidopteran larval pests has been reported following mixed applications of *S. carpocapsae* with chemical insecticides (Ishibashi, 1992). Some pesticides have been shown to enhance *S. carpocapsae* activity (Ishibashi & Takii, 1993), although Gaugler and Campbell (1991) concluded that increased sinusoidal movement did not necessarily enhance host-finding behaviour and thus control potential. The mixing of nematodes and chemicals can offer a better economic return for the grower, with one spray offering maximum pest control. In the current study, only exposure to spiromesifen produced an infectivity level (14.4%) potentially too low for practical use or value. The other chemicals (imidacloprid, thiacloprid and pymetrozine) produced acceptable levels of infectivity (24, 18 and 17.5% respectively).

For the development of a successful IPM system, simultaneous use of insecticides and biocontrol agents may be required. For an insecticide to be compatible with a biocontrol agent and be cost effective within an IPM system it is necessary for the mortality of the target organism to be increased when both insecticide and control agent are used over either treatment alone. In the current work, only thiocloprid provided this additive effect.
This study has shown that spiromesifen alone can offer good control of second instar *B. tabaci*. However, with increasing insecticide resistance always a problem, the study has also identified two approaches to the successful integration of chemical insecticides with the entomopathogenic nematode *S. carpocapsae* to control B. tabaci. Firstly, the insecticides thiacloprid and pymetrozine can potentially be applied simultaneously with *S. carpocapsae* and secondly applying *S. carpocapsae* to plant foliage previously treated with thiacloprid can lead to increased mortality of *B. tabaci*.

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Annex I: : A Standard Operating Procedure (EFF/468 Working with Leptinotarsa decemlineata (Say), the Colorado potato beetle

INTRODUCTION
This SOP describes the work practices involved in the handling and culturing of quarantine chrysomelid beetle species such as *Leptinotarsa decemlineata*. It should be read in conjunction with the general SOP EFF/408 'Working with quarantine organisms (Block 29 and 02F01)'.

L. decemlineata is an A2 quarantine pest for EPPO (see OEPP/EPPO, 1981) and is also EC listed in annex 1B of 2000/29/EC. It attacks potatoes and various other cultivated and wild solanaceous plants. Potatoes, tomatoes and aubergines are at risk wherever cultivated throughout the EPPO region. Wild solanaceous species occur widely and can act as a reservoir for infestation. Adults and larvae can be easily transported on potato plants and tubers, and in all forms of packaging and transport. Fresh vegetables grown on land harbouring over wintered beetles are common means of transport in international trade. The main means of natural spread of the beetle over large areas is by wind-borne migration, particularly of the spring generation. Adults can also be carried over long distances in seawater. *L. decemlineata* is also suspected of spreading several potato diseases, including *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. sepedonicus.

Only personnel who have received training, read the appropriate protocols and signed the Quarantine unit register to confirm compliance, may work unsupervised with these organisms.

Import Procedure and Audit Requirements
The beetles must be ordered from a reputable source, which is approved by the CSL licensing team. The relevant paperwork must be exchanged before proceeding with the order e.g. copy of the Letter of Authority, or the Licence if also required.

The beetles must be contained in unshatterable screw top, sealed, clear jars within sealed plastic boxes. These boxes must be placed in a sealed shatterproof box. A copy of the Letter of Authority must be clearly fixed to the outside of the package to allow it to proceed through Customs. CSL's designated shipping agent must be used in addition to the delivery company (Fed-Ex, DHL etc) to ensure that the consignment is tracked and accounted for at all times.

Beetles should be sent in their lowest risk life stages e.g. eggs or 1st or 2nd stage larvae, to minimise the risk of survival in case of an unforeseen event occurring to the consignment.

On arrival at the licensee's premises, the Senior Plant Health and Seeds/Central Science Laboratory licensing team must be notified. The Senior Plant Health and Seeds Inspector or a member of the Central Science Laboratory licensing team will count and record the number of beetles imported.

A log recording the dates and numbers of beetles introduced or removed (including temporary removal of beetles), and bred must be kept with the beetles' containers - so that all individuals can be accounted for at any time. Numbers of eggs and early instar larvae may be difficult to quantify accurately, but as soon as this is possible detailed records will be kept on the containers themselves, and also in a Logbook.

Numbers of individuals of the different life stages will be kept to the minimum necessary to both maintain a viable culture and provide the experimental specimens. Any individuals surplus to these needs will be killed in the prescribed manner (see point 7), and the details noted in the logbook.

Destruction of live beetles, larvae and eggs not required for culturing or experimental purposes, will be by initially freezing them in a sealed container at -18°c +/-2°c for at least 48 hours, before transferring them to 70% ethanol. The remains of all adult beetles must be retained for inspection and counting by an authorised officer of the Secretary of State or of the Welsh Assembly Government e.g. the Plant Health and Seed Inspector or a member of the Central Science Laboratory licensing team; remains of the other life stages can be disposed of from the alcohol after 1 week.

The designated freezer used to kill any life stage of the beetle must be continuously monitored, and checked once every weekday to ensure that it is maintaining a temperature of -18°c +/-2°c. Any deviation from the parameters must be noted in the logbook, and the beetle material frozen for a further 48 hours, or immediately transferred to 70% ethanol.

ESCAPES/ ACCIDENTAL RELEASE – CONTINGENCY PLANNING
All life stages of the beetle will be kept in clip close plastic boxes within a securely closed plastic tank. These boxes have already been tested by EBGE staff to ensure that In the event of a box being dropped it will not burst open, as long as all 4 clips are correctly closed. After working on a box, the clips are double checked to make sure that they have closed correctly.

All work that involves opening a box that contains adult beetles must be done within a bug dome, or a transfer cage, to provide an extra layer of containment. The number of adults per box will also be limited to a maximum of 10.
If there was an accident and the secure perspex tank was broken or damaged e.g. dropped on the floor, each batch of beetles would still be contained within their individual boxes.
In the event of an escape within the room, in spite of all the precautions taken, the room would be sealed and steps taken to allow the fumigation of the room with a suitable pesticide as advised by the CSL Plant Health Consultants.
The size of the culture will be reviewed regularly, and kept as small as is feasible. It is expected that ~250 adults will be needed per trial, with possibly 6 trials conducted over a period of about 6 months. At any one time there is unlikely to be more than 300 adults present, probably less.

SAFETY
This document should be read in conjunction with SOP EFF/409 'Required standards for laboratory safety, tidiness and equipment'. Before beginning any experiments with Colorado beetle, consult the relevant EBGE COSHH assessments.

Particulate matter produced by insects (e.g. scales and frass) can be a cause of allergies. Workers should read the HSE leaflet Respiratory Sensitisers: a guide for employers (see enclosure). If at any time workers suffer symptoms that may be attributable to the organisms they are working on, they should immediately cease work and inform their line manager. Battery operated pooters should be used as appropriate; orally aspirated pooters should not be used under any circumstances.

Sensitisation to potato plant sap has been previously noted (Paul Bartlett, personal communication), so a lab coat, safety specs and disposable nitrile gloves should be worn when cutting up the plants.

Access to the facilities in Block 29 is restricted to authorised personnel, therefore staff working alone are particularly at risk in the event of accidents. Alarm buttons that will summon a first-aider, or security out of normal hours, are provided in all rooms in Block 29. These should be pressed immediately on feeling unwell.

Experiments involving the spraying of adjuvants should only be conducted within normal hours. Pesticides should NEVER be applied in Block 29.

MATERIALS
CPB holding cages
CPB transfer cage / bug-dome
Modified plastic boxes with tight fitting lock-over lids e.g. "Addis Clip & Close"
Modified plastic mineral water bottles
Modified plastic screw top containers
Food plants e.g. potato (Solanum tuberosum L.)
tomato (Lycopersicon esculentum L.)
aubergine (Solanum melongena)
Water
Large syringe
Cotton wool
35mm plastic petri dishes
Camel hair brush
Plastic stoppers
Modified plastic petri dishes
Plastic funnel
Spoon
Plastic drinking straws
Scissors
Permanent marker
Large white tray with high sides
Sandpit sand
Horticultural loam
Blue roll
Sealable bags
Filter paper
Plastic mesh
Teepol

PROCEDURE
Collect the required plants and leaves of e.g. potato, aubergine or tomato, from Glasshouse 34DG block.

N.B. ONCE YOU HAVE ENTERED THE INSECT QUARANTINE AREA OF BLOCK 29, YOU ARE PROHIBITED FROM RE-ENTERING THE GLASSHOUSE COMPLEX UNTIL THE FOLLOWING DAY.
The first person to enter the facility each Monday is responsible for emptying the waste freezers, before any other work is started. No fresh waste can be added to the bags.

ENVIRONMENTAL CONDITIONS
Temperature: 26.5ºc +/-2ºc
RH: 55%
14hr Light: 10hr Dark
Whenever a container needs to be opened, it must be done within a bug-dome, or ideally the specially designed transfer cage. This provides an additional layer of containment in which to catch any flying beetles, if there was an escape. Larval boxes must be placed in a high-sided white tray before being opened.

1) ADULT HOLDING AND BREEDING BOXES IN 29PG05/6/7/8
Each box can hold ~10 adults, and is lined with damp blue roll.
Adult beetles can survive without food for approximately 10 days. Food should only be added when the box is to be used for breeding. Food should be changed daily, and can consist of leaves/small plants of potato, aubergine or tomato. Cut the stem just above the soil line, and wrap the cut surface in damp blue roll, wrapped in parafilm, to prevent wilting. Or add individual leaves.
Add a small petri dish containing wet cotton wool, as a water source for the holding boxes.
Beetles require a 'lead in' time of approximately 5 days in which to feed and mate before the females begin laying eggs. Setting up a breeding box food on a Tuesday should cause egg laying to commence on the following Sunday/Monday. For a box 10 x 10 x 8 cm add two pairs of adults (the posterior end of the last ventral segment is depressed in the male).
Each breeding box requires extra blue roll, or the addition of damp filter paper, for the adults to lay eggs onto.
Each female can lay up to 2000 eggs over several weeks

2) EGG REARING
The box should be checked twice daily on weekdays, and once a day at weekends, as required.
Yellow/orange oblong eggs are laid in groups of 20-30.Remove clusters of eggs by cutting out the paper (or plant material) that they are attached to, before the adults can eat the eggs.
If fungal growth is a problem, sterilise the surface of the eggs in a very weak bleach solution, and rinse thoroughly.
Place the eggs and/or their substrate onto damp filter paper in a fresh larval box and allow to hatch.
At 26.5ºc +/-2ºc 55% RH eggs should hatch after 3-4 days.
Eggs can be held at 12ºc to delay development or synchronise hatch.

3) LARVAE
Examine the larval rearing box daily during the week, and change the damp blue roll or transfer the larvae to a fresh box as required. Boxes containing first instar larvae should be covered with opaque paper, to encourage them to feed rather than migrate towards the light. Cannibalism during the 1st instar is particularly common at high temperatures with a dry atmosphere, so ensure that the blue roll remains damp.
First instar larvae remain clustered around the egg mass, eating the egg capsules before starting to eat the leaves. The head and pronotum are entirely black.
Second instar larvae begin to disperse to the terminal buds, their head and pronotum are also entirely black. The width of the head capsule can be used to help differentiate between each larval stage (Boiteau & Le Blanc, 1999)
Third instar- anterior margin of the pronotum is orange/brown.
Fourth instar larvae will attack the petioles and stems. At this stage about half the pronotum is light brown anteriorly, black pigmentation disappears, activity drops and they assume a curved shape.
25ºC +/-2ºc gives a larval development time of ~20 days.

5) PUPATION
Place the fourth instars in a pupation box containing 7 cm of a damp loam/sand mixture (3:1 ratio). Add some potato leaves.
Larvae will burrow down a few centimetres, and remain in a pre-pupal stage for several days until they moult, revealing a pupa. This can be sexed, if required – the seventh ventral abdominal segment is depressed in the males. In the females this segment is divided by a dark central line.
Adults should begin to emerge after 5 to 7 days.

6) ADULT COLLECTION TECHNIQUES
Check for emergence each day, and gently collect the individuals in a plastic tub. Teneral adults are frail and must be protected from physical damage.
Transfer them to an adult holding box, setting up a new one each week or as necessary.
The lifecycle is completed in 28-30 days at between 26.5 and 25ºc +/-2ºc.

7) UNWANTED INSECTS

7.1)Destruction of live beetles, larvae and eggs not required for culturing or experimental purposes, will be by initially freezing them at -18°c +/-2°c for at least 48 hours, before transferring them to 70% ethanol. The remains of all adult beetles must be retained for inspection by the Plant Health and Seed Inspector; remains of the other life stages can be disposed of from the alcohol after 1 week.

8) CLEAN UP PROCEDURES

8.1)Empty the remains of the plants and the peat/sand mix into red autoclave bags, and place these and the empty pots in the freezer in 29PG04.
8.2)Place any plastic, tape or paper waste in a yellow clinical waste bag, and place the bag and the empty large petri dishes into the large freezer in the laboratory.
8.3)Bags that are only partly full can be left open in the freezer, and more waste added on the next culturing day.

9) WASTE DISPOSAL

9.1)The first person to enter the facility each Monday is responsible for emptying the waste freezers, before any other work is started. NO FRESH WASTE CAN BE ADDED TO THE BAGS.
9.2)Yellow clinical waste bags must be sealed with a plastic zip tie; red autoclave bags should be tied tightly by knotting the neck.
9.3)Clinical waste should be placed by the entrance to the facility, and FM Helpdesk notified to arrange immediate collection.
9.4)Autoclave bags must be placed in the red plastic bin by the entrance to the facility, and the lid put on firmly. The bags will be collected by a member of the glasshouse staff and autoclaved.
9.5)Plant pots and trays should be placed by the side of the bin, to await collection and cleaning by a member of the glasshouse staff

DOCUMENTS/RECORDS REFERRED TO

SOP EFF/408, Working with quarantine organisms (Block 29 and 02F01)
SOP EFF/409, Required standards for laboratory safety, tidiness and equipment

Boiteau, G. & Le Blanc, J-P.R. (1999) Colorado potato beetle life stages
http://res.agr.ca/lond/pmrc/report.beetle.html

HSE leaflet Respiratory Sensitisers: a guide for employers (a copy is held in the Safety Information File in 02G08)
COSHH EBGE/015/0~ Handling and culturing insects and other invertebrates

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Scientific papers produced from this project:

1) Cuthbertson, A.G.S., Mathers, J.J., Northing, P., Luo, W. and Walters, K.F.A. (2007). The susceptibility of immature stages of *Bemisia tabaci* to infection by the entomopathogenic nematode *Steinernema carpocapsae*. *Russian Journal of Nematology*, **15**: 153-156.

2) Cuthbertson, A.G.S., Mathers, J.J., Northing, P., Prickett A.J. and Walters, K.F.A. (2008). The integrated use of chemical insecticides and the entomopathogenic nematode, *Steinernema carpocapsae* (Nematoda: Steinernematidae), for the control of sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Science*, **15**: 447-453.

3) Cuthbertson, A.G.S., Blackburn, L.F., Northing, P., Mathers, J.J., Luo, W. and Walters, K.F.A. (2009) Environmental evaluation of hot water treatments to control *Liriomyza huidobrensis* infesting plant material in transit. *International Journal of Environmental Science and Technology*, **6**: 167-174.

4) Cuthbertson, A.G.S., Mills, K., Wonter-Smith, T., Blackburn, L.F., Northing, P. and Walters, K.F.A. Evaluation of alternative chemicals to methyl bromide for fumigation of quarantine pests in transit. In draft.