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Biopesticides for the control of storage insect pests

by

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1. ABSTRACT

The UK grain industry stores many tonnes of grain each year. Currently, chemical insecticides are used to protect this grain from infestation by stored product insects and mites. Only a limited number of products are available for application and there are concerns about safety, resistance and environmental impact of these conventional pesticides. In principle, the biological control of storage pests could overcome these problems. A previous LINK funded project (LK0914) established the potential for biological control of storage pests in the UK, using an insect-specific fungus *Beauveria bassiana*. The current project (LK0967) has investigated ways to increase the efficacy of the fungus and has also undertaken research to establish that mass production of high quality conidia (asexual spores) is possible, to ensure that formulations have a good shelf-life and has sought feedback from stakeholders with regard to this novel control method.

The work has concentrated on two different fungal isolates, both of which were found from insects in UK grain stores. The main objectives of the current project were:

1. To improve the efficacy of the insect-specific fungi when in contact with insects.
2. To improve the delivery of the insect-specific fungus to the insects.
3. To ensure the consistency and the safety of the product.
4. To ensure that the biopesticide product has wide acceptability and is effective under practical use.

Optimisation of production methods, formulation and delivery systems has resulted in prototype formulations that have good efficacy against a range of storage insect pests under conditions that are likely to be found in UK grain stores. Valuable information on the viability and efficacy of a range of formulation types was obtained, together with studies on the likely effect of the formulations on insect behaviour. Pilot scale trials using three species of stored product beetle have shown that significant levels of control can be achieved. Successful mass production of the conidia is necessary if the biopesticide is to be considered at a commercial level. This project has developed methods to ensure that mass production of the conidia at an industrial scale is possible. During the course of the project, the views of stakeholders and end-users were sought. Specific concerns were addressed and the feedback was used to focus the research aims. The registration and safety issues for the use of a biopesticide were also studied. This project has made a significant contribution to the development of a biopesticide as a structural treatment for grain storage areas in the UK.

2. SUMMARY

Currently, chemical insecticides are used to protect grain in storage from infestation by stored product insects and mites, but only a limited number of products are available for application. However, there are concerns about safety, resistance and environmental impact of these conventional pesticides. In principle, the biological control of storage pests could overcome these problems with conventional chemical agents. A previous Sustainable Arable LINK funded project (LK0914) established the potential for biological control of storage pests in the UK, using an insect-specific fungus *Beauveria bassiana*. The fungus germinates on and penetrates through the insect cuticle, ultimately resulting in the death of the insect. Project LK0914 (whilst providing evidence for the potential effectiveness of *B. bassiana* against stored product insects) also highlighted areas that required further research. These covered both technical areas, for example methods to improve the efficacy of the fungus, and also related to the practical use of the biopesticide product and the potential barriers to uptake of these novel control agents by key stakeholders. These areas were addressed in the current project, which has examined the potential of a biopesticide based on *B. bassiana* as a structural treatment for UK grain stores. The project has focussed on two different isolates of *B. bassiana*, IMI 389521 and IMI 386243, both of which were isolated from insects found in UK grain stores.

The main objectives of the project were:

1. To improve the efficacy of the fungus when in contact with insects.
2. To improve the delivery of the fungus to the insects.
3. To ensure the consistency and the safety of the product.
4. To ensure that the biopesticide product has wide acceptability and is effective under practical use.

2.1. Objective 1. To improve the efficacy of the fungus when in contact with insects

Beauveria bassiana occurs naturally in UK grain stores, and has been shown to have notable potential for the control of various storage insect pests. However, the control was only satisfactory when the tests were undertaken in laboratory bioassays and, especially for the beetle pests, with high humidity for the first 24 hours. To ensure optimal efficacy in practical situations, it was necessary to improve the germination of the fungal conidia and their penetration into the wide range of pests that need to be controlled, under conditions that represent those encountered in UK grain stores.

The first part of this study aimed to improve the pathogenicity and viability of *B. bassiana* isolates by manipulating the conditions under which the conidia are mass-produced. This was achieved by

subjecting the conidia to different degrees of water stress and determining the effect on production levels, viability and pathogenicity. The effect of four different rice treatments on the production level of conidia was assessed with eight different fungal isolates, which had previously been shown to be pathogenic to the saw-toothed grain beetle, *Oryzaephilus surinamensis*. Generally, fewer conidia were produced per gram of conidiated rice when the rice had received no prior treatment and therefore, had the lowest moisture content. The other three treatments resulted in similar levels of conidia produced for each isolate. The viability of the conidia produced for each of the four treatment methods and the ability of the conidia to germinate at lower water activities (corresponding to lower relative humidities) was assessed for two of the isolates. Isolate IMI 389521 proved to have better viability than isolate IMI 386243, showing no significant decrease in germination over time. The production method did not affect the pathogenicity of the conidia at a low humidity; treatment with conidia produced by the four methods resulted in similar levels of insect mortality, which was very low. To ensure that the production methods had not diminished the ability of the isolates to cause insect mortality, conidia of isolate IMI 386243 produced by the four production methods were tested under conditions of high humidity for the first 24 hours. Good levels of mortality were achieved under these conditions; conidia produced under conditions with the greatest water stress had the higher level of pathogenicity. This study demonstrated that the production method can have an effect on the level of mortality caused by the conidia.

It has previously been demonstrated that some stored product insect species have an innate tolerance against infection by *B. bassiana*. Possible reasons for this were examined. It was demonstrated, using scanning electron microscopy, that quantitative and qualitative differences in adherence and germination of conidia could be observed between a susceptible species, *Oryzaephilus surinamensis*, and a tolerant species, *Tribolium confusum*. At each of the post-treatment periods *O. surinamensis* had a greater number of conidia adhering to the cuticle. This species has a greater number of setae, particularly on the ventral abdomen, and the presence of these may have assisted with adherence of the conidia. Germinating conidia were observed more frequently on the cuticle of *O. surinamensis* than for *T. confusum*. The number of conidia found on both species decreased over time, possibly as a result of grooming activities. The findings suggest that conidia that have not germinated and penetrated the cuticle within the first 24-48 hours are unlikely to remain on the cuticle and play a role in the infection process.

Formulation can play a key role in the efficacy of a fungal biopesticide as it may enhance the infectivity of the fungal conidia and allow a product to be stored over a prolonged period. The viability of conidia of isolates IMI 389521 and IMI 386243 in various formulating agents was examined. A range of formulations were considered including both liquid and dust formulations, various bulking agents and liquid emulsions. In addition, the effect of the formulations on insect mortality was also assessed to determine the optimal formulation for use in further studies. The

formulation studies have shown that in an appropriate formulation, at 5°C, the isolates retained excellent germination over a period of 365 days. In general, IMI 389521 performed better than IMI 386243 with higher initial germination and more reproducible results in experiments. At 25°C, the experiments have shown that, in general, good germination may be achieved after 301 days of storage in mineral-based oils and the powder formulations, with viability remaining above 70%. Vegetable oils, in general, gave excellent results at 5°C. Water-based formulations such as 0.05% Tween 80 were not suitable for either isolate, as viability was lost very rapidly at 25°C and less rapidly at 5°C. There was also an apparent isolate difference when exposed to the emulsifier, Codacide. IMI 386243 lost viability rapidly at 25°C, whereas, this effect was not seen with IMI 389521. The oil formulations resulted in greater mortality of *O. surinamensis* in comparison with the water-based Tween 80 formulation. This reflects previous studies that have shown that oil-based formulations may cause higher mortality than water-based formulations. The powder-based formulations also showed potential. In conclusion, based on the viability and efficacy results, oil- and powder-based formulations look to be good candidates for future use as commercial mycoinsecticide formulations.

These studies have resulted in the development of production methods and formulations that result in high levels of insect mortality without the need for a period of high (close to 100%) humidity for a range of storage beetle pest species.

2.2. Objective 2. To improve the delivery of the fungus to the insects.

Optimising the efficacy of the fungus when it is in contact with insects is necessary for success but is not wholly sufficient: it is essential to ensure that the right amount of the agent is brought quickly enough into contact with the target insects, which may be hiding in cracks and crevices, to ensure control of a pest population. Evidence that this requires further research was obtained in the previous project where it was found that insect mortality was high only when insects were either rolled in conidia or were sprayed directly with them. Very low mortality was observed when insects were left on a treated surface. Improving the delivery of the conidia can be achieved in two ways 1) by moving the insects to the conidia and 2) by moving the conidia to the insects.

Moving the insects to the conidia can be achieved in two ways. Firstly, a repellent could be used to treat cracks and crevices to remove insects from these locations in order to make contact with treated surfaces. Secondly, the insects can be attracted to areas where the conidia are present. Both of these methods were investigated.

Following a literature review of possible repellents that could be used in the grain store environment and that satisfied the criteria of ease of application, low mammalian toxicity, and ready availability and registered for similar usage, two compounds, diatomaceous earths and

pyrethrins were chosen for further investigation. The repellency and mobility effects of pyrethrins and a diatomaceous earth were assessed against adult *Oryzaephilus surinamensis* by recording insect behaviour in response to filter paper halves treated with each compound. DEET (N, N - Diethyl-meta-toluamide), a known insect repellent was used as a positive control. There was no significant difference in the time spent on the untreated halves and those treated with the pyrethrins and the diatomaceous earth, indicating no repellent effect. The beetles did however spend significantly less time on the halves treated with DEET. There was also no significant difference in the distance travelled and velocity of beetles on the untreated halves and those treated with DEET and pyrethrins, suggesting that these compounds did not affect mobility. The beetles did, however, travel significantly shorter distances and were slower on the DE treated half compared to the untreated half and when both were compared to the other treatments, suggesting that the DE reduced insect movement even when away from the treated surface. The ability of DE and pyrethrins to remove insects from refuges was examined by creating artificial crevices containing the test compound. At the concentration tested the diatomaceous earth, Silico-sec, reduced the number of insects that were present in the refuge. This was noticeable one hour after the insects were introduced to the arena. Pybuthrin also reduced the number of insects present in the refuge, but not to the same extent as the diatomaceous earth. The work has shown that, on a small scale it is possible to reduce the number of insects present in a refuge at a given time. However, the ability to achieve this at a larger scale remains to be determined and practical issues with regard to treatment of all potential refuges may preclude this as a practical measure to improve uptake by the insects.

An alternative method to improve the contact between the insects and the conidia is to attract insects to areas where the conidia are present. Much work has been undertaken on developing attractants for stored product beetles. It should, therefore, be possible to attract insects to a bait station containing conidia for use in a lure and kill strategy. Treatments used bait stations either with or without dry conidia powder of isolate IMI 389521. In addition, to determine whether the presence of an attractant lure would improve contact with the conidia, a lure developed to attract several species of stored product beetle was placed in some of the 'bait stations' with and without the conidia. There was a significant difference in the mortality of *O. surinamensis* between treatments with and without the conidia in the bait station; mortality was significantly higher in treatments with the conidia. Mortality in the treatment with the lure in the bait station with the conidia was significantly greater than for the conidia without the lure. This study has shown that insects will enter an area where the conidia are present and will pick up a lethal dose of the dry conidia powder. Bait stations containing an appropriate formulation of the fungal isolate, therefore, offer potential of targeted delivery of the conidia to the insects.

The ability to improve contact by moving the conidia to the insects was also investigated by examining the uptake and behavioural responses of *O. surinamensis* to an electrostatically chargeable powder, Entostat™. This is a processed plant wax and has been identified as a potential carrier for active ingredients to be delivered to cracks and crevices in food facilities. Entostat uptake and retention by *O. surinamensis* 24-72 h after exposure to rolled oats mixed with Entostat was quantified. Depending upon initial Entostat concentration in food mixtures, 0.03-0.26 µg powder was extracted from individual beetles 72 h after being transferred from treated to untreated food. SEM images showed that Entostat adhered to all body parts, including joints, between body segments, and at insertions of body hairs. Choice experiments were used to determine whether *O. surinamensis* individuals were repelled by Entostat. In a three-choice experiment with untreated oats, oats mixed with 5% (w/w) Entostat, and oats mixed with 5% (w/w) Entostat and a piece of filter paper containing a beetle attractant, the beetle attractant did not significantly increase the attractiveness of the crack in which it was applied, but the average powder uptake of beetles from cracks treated with the attractant was significantly higher than from the other cracks. The results suggest that considerable amounts of Entostat were taken up even when beetles were offered a choice between treated and untreated cracks. The addition of Entostat, therefore, provided a means by which contact between the insect and the conidia could be improved.

Dispersal of a fungal infection within an insect population is an important consideration to establish maximum effect. After initial infection, conidia may be passed to other uninfected insects by two mechanisms: secondary transmission and secondary cycling. Secondary transmission results from the physical contact with infected live individuals, whereas secondary cycling occurs after contact with dead mycosed insects. The effects of secondary cycling and secondary transmission of a fungal infection on *Oryzaephilus surinamensis* were assessed in the laboratory. Mortality was evaluated at two temperatures (15°C and 20°C) and four relative humidities (70%, 80%, 90% and 100% rh) with different proportions of infected insects. Secondary cycling was more effective at disseminating the fungal infection than secondary transmission, with 20°C and 100% r.h. the most effective conditions for secondary cycling to take place. Secondary transmission had little effect on increasing the mortality of uninfected individuals.

Some storage beetle species have been shown to exhibit refuge-seeking behaviour. This behaviour can limit contact with residual pesticides but may be advantageous for dissemination, both initial and subsequent, of the biopesticide. However, it is possible that insect behaviour may be altered subsequent to infection or at sub-lethal concentrations. In a preliminary study to examine possible effects of infection on insect behaviour, the effect of a lethal and a sub-lethal dose of *B. bassiana* on the refuge-seeking behaviour of *O. surinamensis* was examined. Significantly fewer insects treated with the lethal concentration of *B. bassiana* were present in the

arena at observation points 48 h post-treatment in comparison with the other treatments. The result indicates that insects infected with a lethal concentration of *B. bassiana* were less likely to leave the refuge than insects receiving the other treatments. The number of dead insects found in the refuge was greater than that found in the arena. This could be of benefit in terms of secondary cycling if conditions within the refuge are suitable. It is likely that uninfected insects would enter the refuge containing the dead, conidiating insects, increasing the likelihood of infection.

In order for a biopesticide to be produced and used at a commercial scale it is necessary to mass-produce the fungal isolate to obtain sufficient quantities. The mass production method must result in a product that is of a high quality and that is consistent between different batches. Sylvania Somycel led the development of the mass production method used in this project. During the process, different aspects of production were examined including the substrate, incubation conditions, extraction process etc. Initial studies used both isolates IMI 386243 and IMI 389521. However, the viability and yields of batches of IMI 386243 were found to be more variable than for isolate IMI 389521. This finding, in conjunction with other data demonstrating greater viability of IMI 389521 under conditions of low water activity, and when formulated in different formulation types, led to the adoption of IMI 389521 as the preferred isolate. The establishment of the mass production method in the latter stages, therefore, focussed on isolate IMI 389521. It is important that the fungus can be produced at an industrial scale and that the product meets established quality control parameters if it is to be used commercially. The scaling up of production from the laboratory was, therefore, an important step if the potential of a biopesticide as a structural grain store treatment is to be realised.

The various studies undertaken to achieve this objective have demonstrated that 'bait stations' offer a potential method for dissemination of the fungal conidia, that the addition of Entostat may improve the uptake of the conidia and that secondary cycling may further spread the infection within an insect population. In addition, a mass production method was established.

2.3. Objective 3. Consistency and safety

An essential part of the project was to show how the safety and quality of the putative biopesticide could be established such that there will be no future technical barriers to its subsequent registration. This included establishing the information that will ultimately be needed for subsequent registration of the biopesticide by industry. In addition, a representative from the Chemicals Regulation Directorate (CRD; formerly the Pesticide Safety Directorate (PSD)) was appointed to the Project Management Group to monitor project progress and provide advice. Discussions with representatives from CRD, particularly with regard to efficacy testing were also held in the latter stages of the project. An overview of the data requirements for a registration package as they currently stand was produced.

The consistency of the mass-produced isolate was confirmed by producing different batches of the chosen isolate and analysing them for compliance with specification standards. The quality of mass-produced conidia by Sylvan-Somycel throughout the project has been very high and the yield has increased with further refinements to the mass production technology since initial laboratory scale mass production experiments were conducted. The particle sizes of IMI 389521 need to be reduced, in general, for good particle suspension in oil formulations and effective spraying. Ideally, droplet sizes for fine oil sprays are 50-100 µm. For dust formulations, particle size is less of an issue for application. The method of application has an important bearing on the ideal size of particle for application. Viability of conidia for IMI 389521 and IMI 386243 from the optimized mass production runs was excellent.

The growing interest in the exploitation of micro-organisms as biological control agents in agriculture has raised concerns about the safety of these organisms. Consequently there is a need for a risk assessment to determine the level of hazard involved and an appreciation that any intervention in an ecosystem will have an impact. Compared with chemical pesticides, mycoinsecticides have features that provide ecologically sound pest control. They are selective to varying degrees, suitable for integrated management techniques, provide an extended period of control by remaining within the environment, and are biodegradable and fundamentally safe. A review within this project of the currently available literature on *B. bassiana* concluded that fungal biocontrol products using *Beauveria* species have a long history of use and have been well tested, but there have been no serious detrimental cases reported. Any potential fungal biocontrol agent will have to undergo testing before it can be released on the market ensuring that all safety issues are covered. However, all the evidence to date indicates that metabolites produced by *Beauveria bassiana* show no risk to humans, animals or the environment.

Finally, as regulatory approval of a biopesticide will require assessment of possible risk due to non-target effects, preliminary studies on the likelihood of non-target effects were made. Some of these, for example carryover on application, are reported for the pilot scale trials under objective 4. Although the biopesticide is intended as a structural treatment, non-target effects would include any effect on the grain in store post-treatment. In this part of the project, the potential effect of the presence of the fungus on the germination of malting barley was examined. The dry conidia powder was mixed with barley at different concentrations and held at different temperatures and varying levels of water availability. Adding a seed-coating of *B. bassiana* increased seed germination under the more temperature and water-stressed conditions; this may have been a combination of conidia providing an absorbent, protective barrier which reduced the rate of imbibition and the germination of the conidia, which may have stimulated seed germination directly by the production of beneficial secondary metabolites or indirectly by out-competing inhibitory

microorganisms on the surface of the seed. However, it was observed that at the more favourable seed germination conditions of 16°C and lower moisture levels, a seed-coating of the fungus had an inhibitory effect on seed germination. The results indicated that a seed-coat treatment of dry *B. bassiana* could affect the timing and/or ability of barley seeds to germinate, and should be investigated further. However, at the levels of *B. bassiana* contamination that could occur on barley in treated premises, no harmful effects were noted.

Although further testing of the formulated product would be needed before the product could be brought to market, the evidence currently available would suggest that such a product would be safe to use for structural treatments. The consistency of the conidia produced using the mass production method has been established and this is also a major step towards the development of a commercially viable product.

2.4. Objective 4. Acceptability and practical use

Use of the biopesticide will depend upon its acceptability to all interested parties and proof that it will function effectively under conditions that are found in typical storage premises. The factors that might affect uptake by industry were examined through consultations with key stakeholder groups. In order to establish the effectiveness of the biopesticide under practical conditions laboratory tests were undertaken on different surface types to ensure that the formulation developed in this project functioned effectively, regardless of surface type. The findings of the laboratory studies of efficacy were validated in pilot- scale trials under conditions which brought together all the difficulties which have to be overcome in real situations, *i.e.* the need to function on combinations of different types of surface under challenging conditions of temperature and humidity, and deal with real infestations of target insects which may try to avoid treatment. Finally, the versatility of the biopesticide for users, and its acceptability to the potential manufacturer and distributor were assessed.

The development of any novel control method should consider not only the technical obstacles and developments, but also needs to consider how such a product would be viewed by stakeholders when used under 'real-world' conditions. The aim of this step was to identify the likely issues, if any, that will inhibit take up of a commercial product based on the biopesticide developed within the project. This was achieved by meeting with representatives of each stakeholder group to outline the project aims and progress, to ascertain any concerns and to identify information that ideally the project would need to provide. The main concerns expressed by the majority of stakeholders fell into the following categories:

1. Efficacy - the range of pests that could be controlled with this type of product, whether limitations are imposed by temperature and humidity conditions, the potential shelf life of the product and the length of the residual effect after treatment.

2. Health and safety considerations for operators - the type of formulation was regarded as a key issue with stakeholders expressing concerns over the use of dust formulations due to potential respiratory risks. This was stated to be one of the main constraints to the use of another novel method for grain store pest control, diatomaceous earths. The potential for the production of secondary metabolites was also a concern.
3. Risk of contamination - the key concern was whether there could be a build-up of inoculum and whether the inoculum could pass further along the processing chain, for example, affecting germination of malting barley or taint in final products
4. Use - cost was seen as a key issue for some stakeholders, whilst others would be willing to accept an increased cost (assuming acceptable efficacy) if this led to a substantial reduction or elimination of the use of organophosphate insecticides.

Many of the concerns raised were similar for all of the groups. The information was used to guide the experimentation within the project and data has been generated that can specifically address some of the issues raised.

In order to establish the effectiveness of the biopesticide under practical conditions, experiments examined the effectiveness of the fungus when applied to different surface types likely to be found in a typical UK grain store. It is important that efficacy of the biopesticide is retained on all surface types and ideally that the viability of the conidia is maintained to provide a degree of persistency. Viability of the fungus was found to be affected by the type of surface, to which it was applied, with a substantial loss of viability observed on the concrete after only one day. The viability on wood and steel also decreased over the 13-day test period, but not to the same degree as the conidia on the concrete. Only one formulation type was tested in the laboratory and it is possible that other formulations may maintain the viability of the conidia to a greater degree. This was explored further in the pilot- scale trials reported below.

The dry conidia powder, either alone or when mixed with Entostat, resulted in high levels of mortality of *O. surinamensis*. The mortality level on wood was slightly less than on steel or concrete. This may have been as a result of the surface of the plywood, which has small grooves in which the conidia can lodge, perhaps making contact with the insects more difficult. Persistency of the conidia on the various surface types was not explored in this laboratory trial, but the effectiveness of the treated surfaces over time was explored in the pilot scale trials.

The laboratory studies had shown that isolate IMI 389521 has good efficacy against storage beetle pests under constant temperature and humidity conditions and that a good level of efficacy was maintained when the isolate was applied to different surface types. The conditions chosen for the laboratory studies were close to optimal for both the fungus and the insects. To act as an effective

structural treatment in a grain store, it is important that efficacy is maintained for a reasonable period under the fluctuating environmental conditions that would typically be encountered in this situation. It was therefore, important to test the fungus in a larger scale experiment under conditions that would typically be encountered in a UK grain store. For the purpose of this experiment, two formulations were chosen for further investigation. The results from the first pilot scale trial indicated that there were differences in the efficacy of the two formulations. However, the data also showed that the different application methods used resulted in a difference in the concentrations of the two formulations applied to the arenas. To establish whether both formulations should remain as candidates for further testing, it was necessary to examine the effect of the two formulations when applied at similar concentrations. The second pilot scale trial examined the effect of the two formulations of IMI 389521 at two target concentrations on the mortality of three species of stored product beetle when applied to plywood arenas. In addition, the viability of the conidia in the two formulations under typical UK grain store conditions was again assessed. A comparison with a currently registered chemical pesticide, pirimiphos methyl, was also made.

The chemical pesticide, Actellic (pirimiphos methyl) when applied at the recommended concentration caused rapid death of all three species of insect. Large numbers of knock down or dead insects were observed within two hours of the introduction of the insects to the treated surface and 100% mortality was recorded for insects recovered from the rings after 14 days. The biopesticide formulations also caused a significantly greater level of mortality than was observed for the control treatments. The viability of the conidia on realistic surfaces, as determined by the % germination, remained high throughout the trial indicating that under the test conditions isolate IMI 389521 retained the potential to infect insects and may, therefore, have residual activity.

A novel insect control agent for use in UK grain stores should ideally integrate with current procedures for best practice. Therefore, the control agent should form part of an integrated pest management (IPM) approach. The cost effectiveness of any novel agent is a key factor in determining likely future commercial exploitation. The development of the mass production methods within this project and determination of the likely maximum quantity for use of the agent on its own have provided data that can be used to examine the likely costs to produce the agent. The cost of the use of the agent on its own should also be considered with the costs of use in combination with another control method. This may also increase the versatility of use of the biological control agent. It is known that the use of a diatomaceous earth (DE) structural treatment has potential against the saw-toothed grain beetle, *O. surinamensis* and it has been reported that a commercial DE synergises the effect of unformulated conidia of *B. bassiana* against some storage pests. The effects of the biopesticide formulation and DE when used alone at a single concentration were compared to the effect of the combination by exposing *O. surinamensis* in

treated Petri dishes to provide information on the versatility of the biopesticide for potential users. The combination of IMI 389521 and Silico-sec resulted in a significant increase in the overall mortality in comparison with either treatment alone at both 15°C and 20°C. At both temperatures, significant synergism was indicated and this was particularly apparent at 15°C.

This part of the project has used the data provided from the three previous objectives and through the pilot scale trials has demonstrated that a biopesticide based on *B. bassiana* has great potential as a structural treatment under typical UK storage conditions. Establishing the key concerns that stakeholders may have in relation to this type of product has enabled data for many of these concerns to be obtained within the course of the project and for this information to be fed back to the stakeholders.

2.5. Conclusions

This study has successfully achieved all four main objectives of the project. Of particular note, is that enhancement of the production and formulation of the conidia has negated a need for a period where the humidity needs to be close to 100% and the conidia do not have to be directly applied to the insects to achieve good efficacy. In addition, mass production methods resulting in consistent, high quality production of conidia with excellent viability and virulence have been developed and significant control of insect populations under practical conditions has been demonstrated.

This research has made a significant step towards the development of a biopesticide, based on *B. bassiana*, as a structural treatment in UK grain stores. In addition to overcoming the main technical obstacles, information has also been collected on the concerns and issues that the development and use of a biopesticide may generate. The research undertaken within this project has, as far as was possible, addressed these concerns and generated important data to demonstrate that this type of product can be used in a practical situation to achieve good levels of control. Information has also been gathered on the registration process for a biopesticides. This will be important in the future development of the product and provides a clear foundation to determine the further studies that would be necessary.

The project has demonstrated that a biopesticide based on *B. bassiana* has potential for control of stored product insects in UK grain stores. Candidate formulations have been identified. However, further work will be needed to fully establish the most appropriate formulation. The mass production process has been optimised, but until the most appropriate formulation and dose rate have been established, it will remain to be seen whether cost-effective production can be realised. The project has made significant progress in the development of a novel structural treatment that would be a benefit to UK farmers.

3. TECHNICAL DETAIL

3.1. Introduction

Cereals are an important component in the average UK diet and are similarly important in livestock feedstuffs. Cereals are stored prior to further processing and during this time are at risk to infestation by insects, which causes quality deterioration and losses. Pesticides are commonly used for control of stored product insects. In 2002, over 9 tonnes of active ingredients were used as fabric treatments to protect the harvest in Great Britain (Dawson *et al.*, 2004a, b). There are concerns about the safety of some insecticides, in particular organophosphates, and recent changes in EU legislation may result in removal of some pesticide products currently in the market place (Pesticides Safety Directorate, 2008). Concerns have also arisen with regard to insect resistance to commonly used products. Resistance has been reported for all insecticide classes for one of more key pest species (Whalon *et al.*, 2008), including stored product insects. With an increasing emphasis on food security, the food industry urgently needs alternative approaches for control of stored product insects.

Alternatives to chemical control agents have been studied. Biological control of storage pests is one of the possible novel approaches that could overcome the problems with conventional chemical agents. While bacteria, fungi, protozoa and viruses all have potential as natural microbial control agents (biopesticides), it is the insect-specific (entomopathogenic) fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* about which most is currently known and which are the best candidates. These fungi are able to infect insects as a result of the conidia adhering to the insect cuticle and germinating. The fungus penetrates through the cuticle, invading the insect haemolymph and other organs, eventually resulting in the death of the insect. Under appropriate conditions, the fungus is able to sporulate on the dead insect, producing new conidia that can infect other insects. Due to their specificity, these naturally occurring fungi have low mammalian toxicity and very low environmental impacts. Therefore, they have potential for use in integrated pest management systems for a wide variety of pest species. Whilst there is much research on the identification of suitable fungal isolates and development of mycopesticides, some are already commercially available. A review of all commercially available products containing entomopathogenic fungi to control a range of insect and mite pest species has recently been compiled (de Faria and Wraight, 2007). Interest in the use of biological control agents (BCAs) has increased and recently an EU policy support action examining registration of BCAs, including entomopathogenic fungi, known as REBECA (Regulation of Biological Control Agents) was completed. This action reviewed the possible risks of biocontrol agents, compared regulation in the EU and the USA and proposed alternative, more efficient regulation procedures to maintain the same level of safety for human health and the environment but to accelerate market access and

lower registration costs (www.rebeca-net.de). The potential for use of BCAs in a wide range of situations is, therefore, increasing as both technical and legislative advances are made.

Research to investigate the potential of entomopathogenic fungi for control of stored product insects has increased over the past decade, with studies examining the potential of both *M. anisopliae* and *B. bassiana* on a range of species. The majority of these studies have examined the efficacy when the fungus is applied to the grain (Lord, 2001; 2005; 2007a, b; Akbar *et al.*, 2004; Vassilakos *et al.*, 2006; Michalaki *et al.*, 2006; Kavallieratos *et al.*, 2006; Cherry *et al.*, 2007; Hansen and Steenberg, 2007; Athanassiou *et al.*, 2008) and there have been few studies on the potential as a structural treatment or under environmental conditions that are appropriate to the UK. A previous Sustainable Arable LINK funded project (LK0914) confirmed the potential for biological control of storage pests in the UK (Cox *et al.*, 2004). Eight isolates of *B. bassiana* were collected from various UK stores and tested against representative species of beetle, moth, psocid and mite, including some resistant to organophosphates. Although 100% kill was obtained against one or more species in 7 days in laboratory tests, the project showed that, under conditions representing those in stores, it would be essential to improve the uptake by the pests of the fungal conidia, their germination and penetration into the pests.

The main objectives of the current project were:

1. To improve the efficacy of the insect-specific fungi when in contact with insects. The hypothesis is that problems with germination and penetration of fungal conidia into storage pests can be overcome by the manipulation of conidial production conditions, elucidating differences between beetle species and applying basic knowledge of formulations.
2. To improve the delivery of the insect-specific fungus to the insects. The hypothesis is that the need to ensure adequate contact between the conidia and the target pest can be achieved by exploiting a combination of insect behavioural traits and novel application technology.
3. To ensure the consistency and the safety of the product. The hypothesis is that by identifying at the earliest stage the regulatory requirements for biopesticide use in storage, and by showing how they can be met, on completion of this project there will be a clear route by which industry can register the prototype product.
4. To ensure that the biopesticide product has wide acceptability and is effective under practical use. The hypothesis is that by taking early and pro-active steps to consult interested parties, the work in this project can be designed to meet their requirements and avoid subsequent problems in uptake.

These objectives were met by completing a series of steps, which are examined in detail in this report.

3.2. Objective 1. To improve the efficacy of the fungus when in contact with insects

Beauveria bassiana occurs naturally in UK grain stores, and has been shown to have notable potential for the control of various storage insect pests (Cox *et al.*, 2004). However, the control was only satisfactory when the tests were undertaken in laboratory bioassays and, especially for the beetle pests, with high humidity for the first 24 hours. To ensure optimal efficacy in practical situations, it was necessary to improve the germination of the fungal conidia and their penetration into the wide range of pests that need to be controlled, under conditions that represent those encountered in UK grain stores. This was achieved in the following three steps. **Step 1.1:** The putative biocontrol agent was optimised for germination, virulence and persistency. This was done by adjustment of the conditions under which it was produced. **Step 1.2:** Different species of storage beetle have different susceptibilities to fungal conidia at the same concentration (Cox *et al.*, 2004). Factors that may influence susceptibility early in the infective process were examined to identify critical stages. **Step 1.3:** Prototype formulations were developed based on the information from the two steps above and from Objective 2 (step 2.1) and Objective 4 (step 4.1) below, together with consideration of how the agent will best be applied.

3.2.1. Step 1.1 Optimization of conidia

Introduction

The efficacy of fungal biological control agents can be reduced if the conidia lose viability or are unable to germinate and penetrate the insect cuticle due to low water availability (Doberski, 1981). Cox *et al.* (2004) demonstrated that a high humidity, and hence high water availability, was necessary to achieve high mortality of stored product beetles with *B. bassiana*. One possible way to improve fungal efficacy is to produce conidia that are more robust and able to germinate at lower water availability. The mass production methodology for fungal isolates is very important as it has implications for the efficacy and storage of the product. It is possible to vary the quality of conidia when mass-producing by manipulating the conditions of mass production. This can lead to more robust conidia that are more tolerant of environmental conditions, but it may also result in fewer conidia being produced. Hallsworth and Magan (1994a) stated that by altering the levels of various carbohydrates (such as polyol and trehalose) in conidia, through manipulation during growth, the efficacy and shelf life of an agent might be enhanced. It was also demonstrated that the alteration of the growth conditions, to induce water stress, resulted in growth of conidia that could germinate under drier conditions (Hallsworth and Magan, 1995). The accumulation of sugars during conidiation may be affected by the level of water stress the fungus is exposed to during growth; in turn, by altering the conditions under which conidiation occurs, these conidia may subsequently be able to germinate over a wider range of environmental conditions (Magan, 2001). Hallsworth and

Magan (1994b) demonstrated that *B. bassiana* conidia grown under such conditions were more effective at lower levels of relative humidity than control conidia produced on normal media when tested against *Galleria mellonella* (Greater Wax Moth).

In order to produce an effective biopesticide, the isolate selected needs to not only induce high levels of mortality in the target insect population, but also be a cost-effective product that can produce an economically viable number of conidia, which can be stored until use and be effective in the target environment. Wraight *et al* (2000) stated that for economic production of conidia, production costs need to be comparable to the cost of chemical insecticides, and this has been a goal for those undertaking commercial development. Wraight and Carruthers (1999) stated that *Beauveria bassiana* has achieved one of the greatest production efficiencies due to its small conidia.

The aim of this study was to improve the pathogenicity and viability of *B. bassiana* isolates by manipulating the conditions under which the conidia are mass-produced. This was achieved by subjecting the conidia to different degrees of water stress and determining the effect on production levels, viability and pathogenicity. In this way, it was anticipated that the conidia would be able to perform better in environments with lower relative humidity.

Materials and methods

1. Production of conidia under different degrees of water stress

Liquid broth was prepared by dissolving 20 g yeast extract (Oxoid, LP002) and 20 g sucrose (BDH, GPR, product number 302994G) in 1 litre of tap water. The broth was transferred into 250 ml conical flasks in 75 ml aliquots. Bungs were placed in the tops of the flasks and covered with tin foil. The flasks were autoclaved at 15 psi, 121 °C for 15 minutes and allowed to cool before inoculation. Conidial suspensions were prepared by gently removing conidia, aseptically, from potato carrot agar (PCA) plates and suspending them in 0.05 % Tween 80 solution. This was done for each of the eight isolates selected (IMI 386243, 386367, 386368, 386605, 386606, 386607, 387297 and 389521). The resulting suspensions were sonicated for three minutes to break up any conidial chains. The conidial suspensions were adjusted to 8×10^6 conidia/ml and an aliquot (1 ml) was transferred aseptically into a conical flask containing the sterile broth. The inoculated flasks were placed onto a rotary shaker at 170 rpm for three days.

Four rice treatments (Tilda basmati rice) prepared with different amounts of water, to simulate different degrees of water stress, were used to produce the conidia as follows:

- Treatment 1 – Rice with no prior preparation and no cooking
- Treatment 2 – Rice washed under cold running water for a minimum of 5 minutes and no cooking

- Treatment 3 – Rice cooked with 300 ml water and 20 ml oil/kg rice
- Treatment 4 – Rice cooked with 600 ml water and 20 ml oil/kg rice.

A non-generic vegetable oil was used. The rice was cooked using a Samsung rice cooker (SJ-420E, 4.2 l capacity) with an automatic cooking gauge, so that the cooking time varied for each treatment. After preparing the rice, the following quantities were measured into 780 x 400 mm autoclave bags:

- Treatment 1 – 200 g
- Treatment 2 – 224 g
- Treatment 3 – 247 g
- Treatment 4 – 292 g

The weights represent 200 g of dry rice and the rest of the weight was water absorbed by the preparation procedure. The bags of rice were autoclaved for 40 minutes at 15 psi, 121 °C and allowed to cool before inoculation. The moisture content (MC) of the rice was determined after autoclaving using a Mettler Toledo halogen moisture analyzer (HG53).

The inoculated liquid broth was removed from the shaker and diluted with sterile tap water (50/50 dilution). From the diluted inoculum broth 30 ml was transferred onto a rice sample. For each isolate one bag from each of the four treatments was inoculated. The inoculum was massaged into the rice to ensure even coverage. The autoclave bags were folded once at the top and stapled. The bags were spread out to give an even layer of inoculated rice and were puffed up to allow aeration. After 11 days the bags were opened and the conidiated rice was allowed to dry at room temperature for 7 days. Samples of the rice were taken after drying. The conidiated rice was suspended in 5 ml Shellsol T and the conidia/g conidiated rice was determined by carrying out haemocytometer counts. The moisture content of the rice at sampling was approximately 10 %. This experiment was repeated on five separate occasions using the same procedure.

2. Assessment of viability on agar with different water activities

Two isolates of *B. bassiana* (IMI 386243 and IMI 389521) that when mass produced resulted in quantities of conidia that would be feasible for scale up to commercial mass production levels, were selected. In this set of experiments, conidia were used from the same four mass production treatments as described in section 1. When conidia were ready to be harvested, a universal of rice+conidia from each treatment was separated and stored at 5°C for 0, 1, and 8 weeks. Conidia were harvested at each time interval and tested for viability on agar prepared at various water activities, which replicated lower levels of relative humidity. Agar was prepared using polyethylene glycol 200 (PEG) using a recipe provided by N. Magan (personal communication). Different concentrations of PEG were added to the agar and the water activity of the agar was tested using

an Aqua Lab Series 3 TE moisture analyser. Media with the following water activities (a_w) were prepared: 0.987, 0.978 and 0.968. The viability of conidia was assessed over three different time points on the different levels of PEG adjusted agar. Agar with 0.987 a_w acted as the control. Conidia of isolates IMI 386243 and IMI 389521 were harvested from the various rice mass production treatments described above, after having been stored for 0, 1 and 8 weeks, using 0.05 % Tween 80 + distilled water adjusted with polyethylene glycol 200 (PEG) to give <0.968 water activity (a_w). A small drop of the conidia suspension was then plated out on Sabouraud dextrose agar (SDA) adjusted with PEG-200 to give water-adjusted growth media at 0.987, 0.978 and 0.968 a_w . The plates were sealed with parafilm and stored at $25 \pm 2^\circ\text{C}$ for 24 h. Conidia germination was then assessed, with a minimum of 300 conidia counted per plate. A conidium was classified as germinated if the length of the germ tube exceeded the diameter of the conidium. For each rice treatment there were one to four germination plates per a_w . The whole trial was carried out on three separate occasions with a fresh batch of rice + fungus produced on each occasion.

Viability was assessed as a percentage, and these values were subjected to an arcsine transformation prior to analysis. Differences between mass production treatments and water activities were assessed at day zero using a two-way ANOVA and Tukey post hoc test. Differences in viability over time were assessed on the 0.987 a_w standard agar and analysed using a one-way ANOVA.

3. Assessment of pathogenicity of conidia

Conidia of isolate IMI 386243 or 389521 produced using the four different production methods were suspended in sterile distilled water containing 0.05% Tween 80 at a concentration of 1×10^7 viable conidia/ml. Suspensions were sonicated and vortex-mixed to ensure an even distribution of the conidia prior to use. Adult *Oryzaephilus surinamensis* (strain Tram) were batched in groups of 20 in 7.5 x 2.5 cm glass tubes. Each batch of insects was treated individually by transferring the insects into a 1.5 ml microcentrifuge tube and adding 1 ml of the conidial suspension. The tube was inverted slowly 10 times and the total length of immersion was approximately 30 seconds. The contents of the tube were poured into a Buchner funnel containing a Whatman No. 1 filter paper and the suspension was removed under vacuum. Each replicate treatment was transferred to individual 9 cm diameter Petri dishes and kept at 20°C , 70% r.h. Control insects were treated with sterile distilled water containing 0.05% Tween 80. Mortality was assessed after 14 days.

Results

1. Production of conidia under different degrees of water stress

As expected, the moisture content of the rice prepared using varying amounts of water differed between the treatments; the rice treatment with the highest amount of added water had the highest moisture content (Table 1).

Table 1. Moisture content of rice treatments after sterilisation by autoclaving.

Treatment	Moisture Content
Rice with no prior treatment	13.57 %
Rice washed for 5 minutes	23.26 %
Rice cooked with 300 ml water/kg rice	30.03 %
Rice cooked with 600 ml water/kg rice	37.24 %

There was no significant difference between conidia/g produced by each isolate ($F=1.57$, $df= 7$, $p=0.15$). However, for the majority of isolates, fewer conidia were produced when grown on the dry rice (Figure 1). A one-way ANOVA showed there was a significant difference between conidia/g produced between rice treatments ($F=3.116$, $df= 3$, $p=0.03$), and a Tukey post hoc showed that the dry rice treatment produced significantly fewer conidia than the 600ml/kg rice treatment (Tukey post hoc $p=0.03$). The other three treatments showed very little difference in the numbers of conidia produced (Figure 1). Isolate IMI 386606 was very variable between replicates, as indicated by the large standard error (SE) bars on Figure 1.

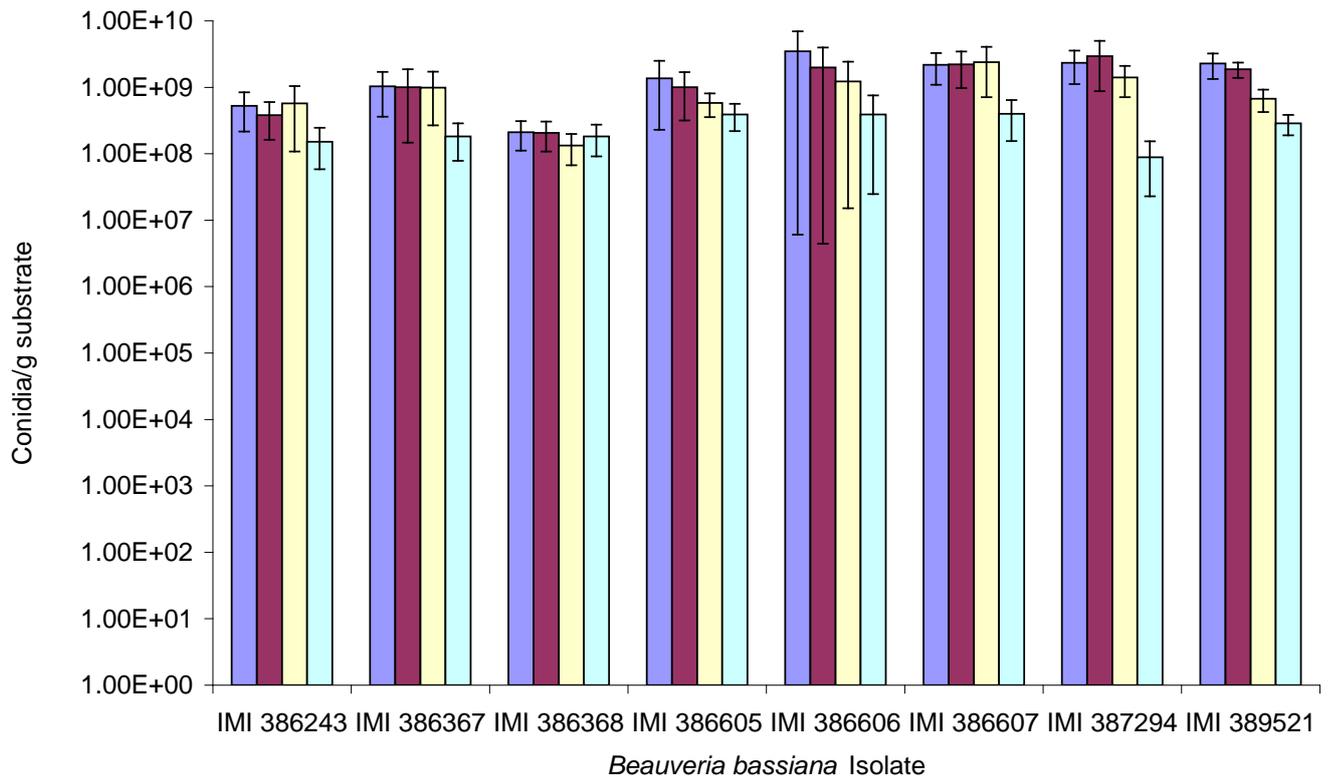


Figure 1. Comparison of conidia/gram of substrate under four different water regimes for eight isolates of *B. bassiana* Key: ■ = 600 ml water/kg rice, ■ = 300 ml water/kg rice, ■ = washed rice, ■ = dry rice.

2. Assessment of viability of conidia on agar with different water activities

The results for IMI 389521 grown on 0.987_{a_w} agar showed that there was no significant drop in viability over time ($F=0.52$, $df=2$, $P>0.05$) (Figure 2). A two-way ANOVA showed there was a significant difference between mass production treatments at Day 0 ($F= 30.31$, $df=3$, $P<0.05$). Germination was highest in the 300 ml and 600 ml mass production treatments, which were significantly higher than the dry mass production treatment ($P<0.05$ Tukey HSD), which in turn was significantly higher than the washed mass production treatment ($P<0.05$ Tukey HSD). The 600 ml treatment was marginally better than the 300 ml, although this difference was not statistically significant ($P>0.05$ Tukey HSD). The 600 ml treatment also showed excellent results at 0.968_{a_w}, as average germination remained above 80% for all time points. At Day 0, however, a two-way ANOVA showed there was no significant difference in germination of the conidia grown on agars at different water activities ($F=2.03$, $P>0.05$) indicating that treatments did not improve or decrease the ability of the conidia to germinate across different water activities. These results show that it is possible for isolate IMI 389521 to germinate at lower water activities using the appropriate mass production method. The isolate, as a whole, performed well over the 8-week experimental period, showing no significant drop in viability.

The results for isolate IMI 386243 were more variable and germination levels were generally lower (Figure 3). At 0.987_{a_w} there was a significant drop in germination over time ($F= 4.98$, $df=3$, $P<0.05$). Germination at weeks 1 and 8 was significantly lower than at week 0 ($P<0.05$ Tukey HSD) and viability as a whole was poorer than for isolate IMI 389521. Apart from the 600 ml / 0.987 water activity, all treatments showed <50% germination after 8 weeks. Germination was consistently poor in all treatments for conidia grown on 0.968 and 0.978 water activities with average germination never rising above 60%. There was no significant difference between mass production treatments on day 0 ($F=1.34$, $df=3$, $P>0.05$) and no significant difference between germination at different water activities on day 0 ($F=1.88$, $df=2$, $P>0.05$).

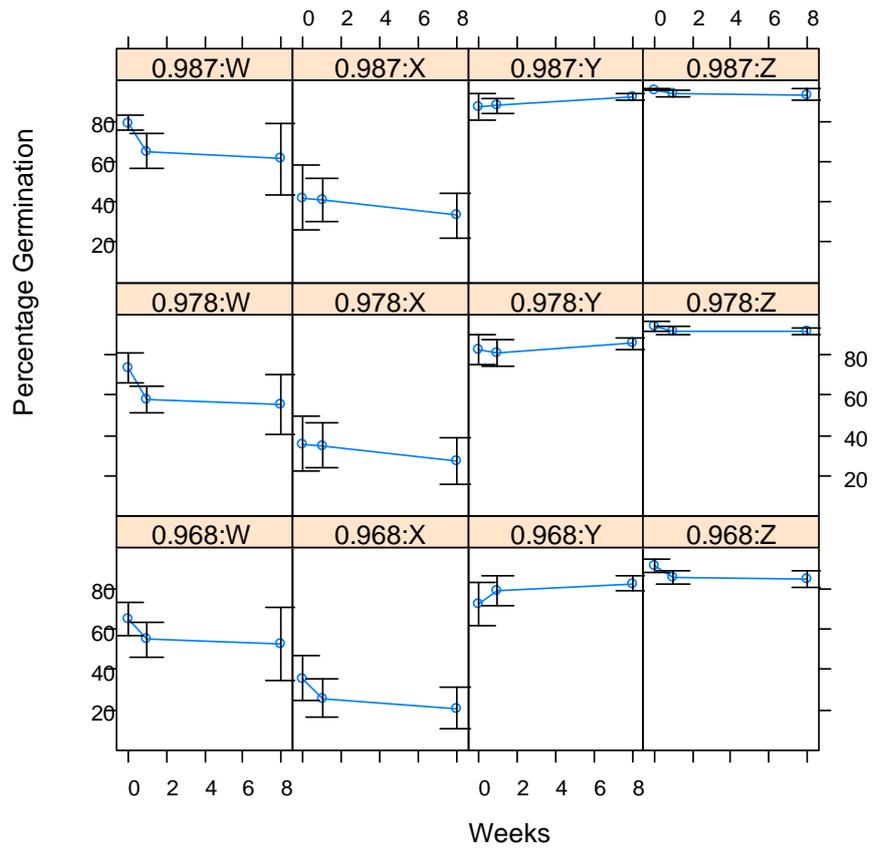


Figure 2. A multi-panel plot of the average percentage germination of IMI 389521 over time in weeks (± 1 SE), categorised by water activity (a_w 0.968, 0.978, 0.987) and mass production treatments (W= dry rice, X= washed rice, Y= 300ml water and Z= 600ml water).

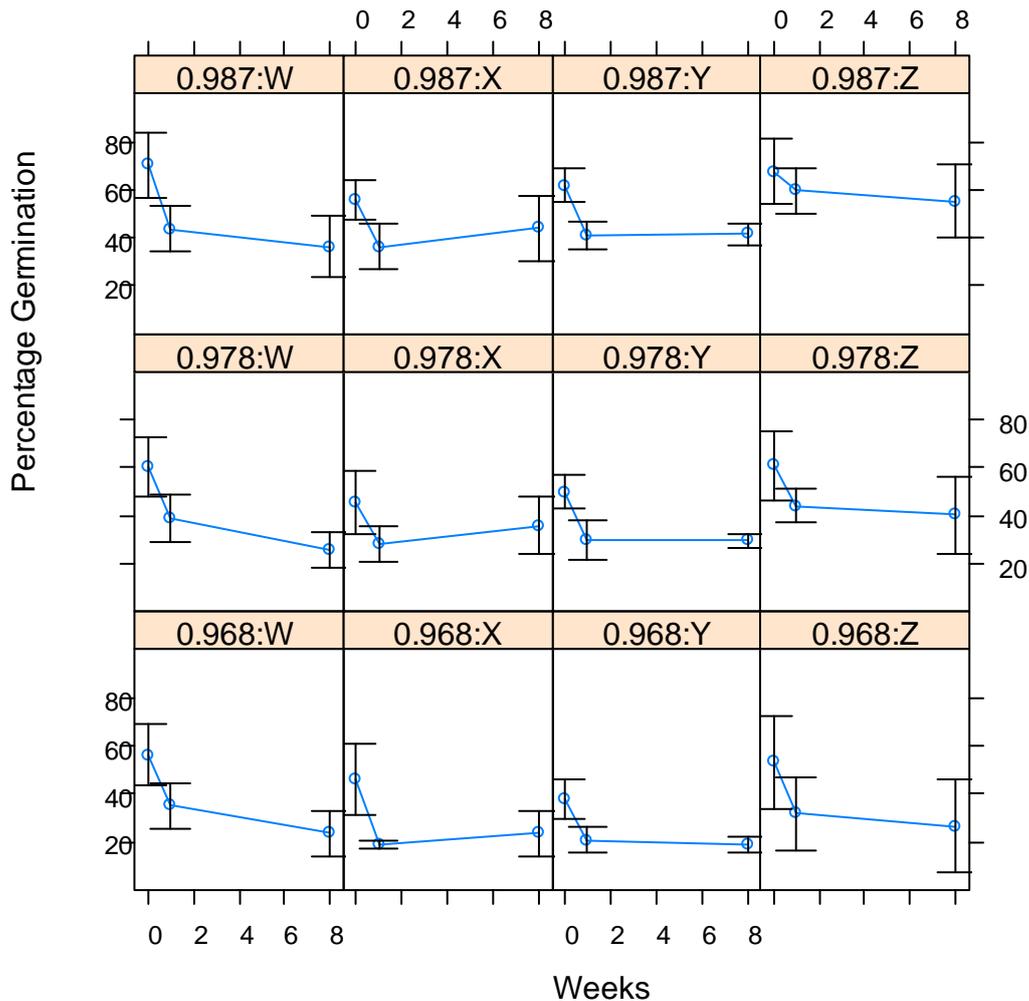


Figure 3. A multi-panel plot of the average percentage germination of IMI 386243 over time in weeks (± 1 SE), categorised by water activity (a_w 0.968, 0.978, 0.987) and mass production treatments (W= dry rice, X= washed rice, Y= 300ml water and Z= 600ml water).

3. Assessment of pathogenicity of conidia

Very low mortality levels were found with both isolates and with all four conidia production methods 14 days after treatment when insects were kept at 20°C, 70% r.h. throughout the experiment (Figure 4). To ensure that the conidia produced were pathogenic to the insects, a further test was carried out using IMI 386243 in which a filter paper moistened with 750 μ l of sterile distilled water was placed in the Petri dish for the first 24 hours of the test. This resulted in much greater levels of insect mortality (Figure 4). Conidia produced using the dry and washed rice treatments resulted in significantly greater levels of mortality than those from the 300 ml and 600 ml rice treatments.

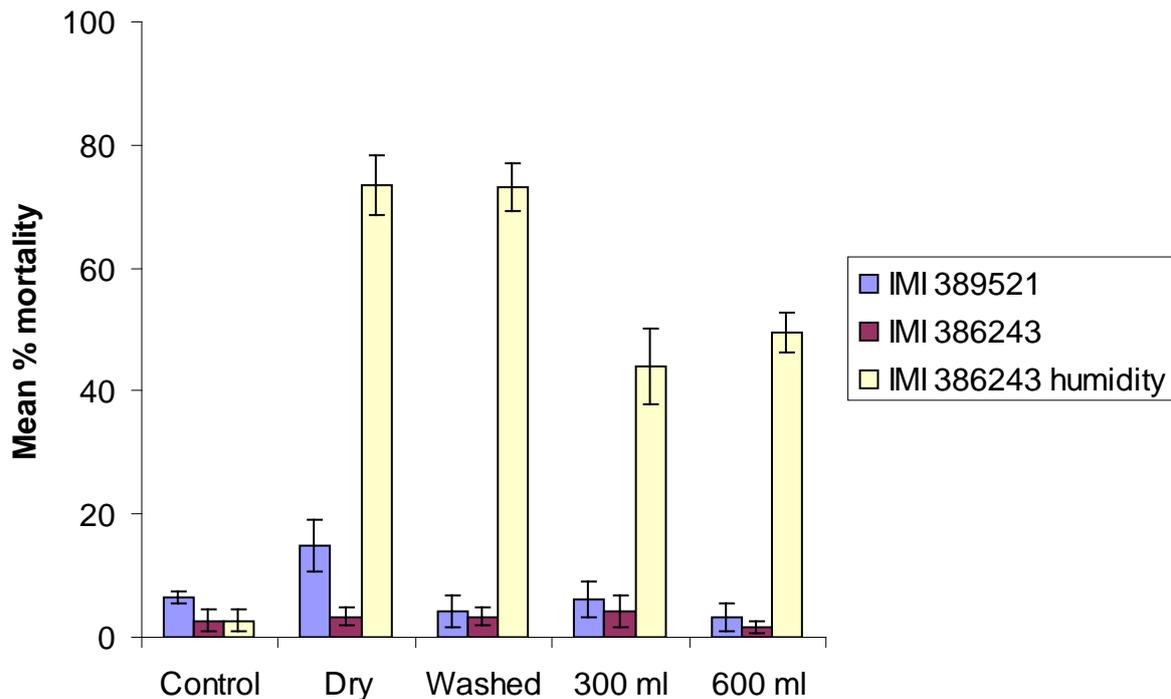


Figure 4. Mean % mortality (\pm se) of *Oryzaephilus surinamensis* (Tram) 14 days after treatment with conidia produced by different production methods. Insects were treated with solutions containing 1×10^7 conidia/ml.

Discussion

This step aimed to optimise conidia in terms of pathogenicity and viability at lower relative humidities. This was done by altering the production method so that the conidia were produced under different degrees of water stress. The effect of four different rice treatments on the production level of conidia was assessed with eight different fungal isolates, which had previously been shown to be pathogenic to the saw-toothed grain beetle, *O. surinamensis* (Cox *et al.*, 2004). Generally, fewer conidia were produced per gram of conidiated rice when the rice had no water in the production method and therefore had the lowest moisture content. The other three treatments (rice washed or 300 ml or 600 ml of water added) resulted in similar levels of conidia produced for each isolate. This indicated that if the pathogenicity and viability of the isolates were improved at lower humidities, the treatment method was not likely to be an impediment to production at economically viable levels. All eight isolates produced fairly similar numbers of conidia and, therefore, two isolates, IMI 386243 and IMI 389521 were chosen for further studies based on previous insect bioassay results (Cox *et al.*, 2004).

The viability of the conidia produced for each of the four treatment methods and the ability of the conidia to germinate at lower water activities (corresponding to lower relative humidities) was assessed. Isolate IMI 389521 proved to have better viability than isolate 386243, showing no significant decrease in germination over time. The 300 ml and 600 ml treatments had consistently high germination, showing little variation between replicates. The 600 ml mass production

treatment performed best overall with excellent germination levels observed at lower water activities (0.968). The poorest mass production method for viability of the conidia proved to be the washed rice, which performed poorly for both isolates. The manipulation of the mass production method, by inducing water stress had little bearing on the performance of IMI 386243. Germination was poor throughout all the treatments and there was no effect observed between conidia grown on different levels of water activity. The mass production treatment of IMI 389521 did have an affect on the germination of the conidia in different water activity treatments; however, the differences were not statistically significant. This indicates that the mass production method did not improve or impede germination across these conditions. It has been previously hypothesised that producing conidia under conditions of water stress, which would apply to the dry and washed treatments, would produce conidia more able to germinate at lower water activities. In these experiments, there was no effect of the production method on the ability to germinate at lower levels of water activity and it appears that isolate IMI 389521, grown under the optimum conditions, performs naturally well at levels of low water availability.

The production method did not affect the pathogenicity of the conidia at a low humidity; treatment with conidia produced by the four methods resulted in similar, very low levels of insect mortality. A test using solutions containing 1×10^8 conidia/ml of isolate IMI 389521 resulted in slightly higher mortality levels (maximum 25%) (results not shown), but these levels were still not acceptable. To ensure that the production methods had not diminished the ability of the isolates to cause insect mortality, conidia of isolate IMI 386243 produced by the four production methods were tested under conditions of high humidity for the first 24 hours. Good levels of mortality were achieved under these conditions and significant differences were found depending on the production method. Conidia produced under conditions with the greatest water stress had the higher level of pathogenicity. Previous studies to examine the effect of manipulation of growth media on the germination and subsequent pathogenicity of conidia (Hallsworth and Magan, 1994a, b; 1995) have shown that the amount of low molecular weight polyols present in conidia increases when the water activity of the growth media is reduced by the presence of different carbohydrate sources. Conidia produced under these conditions were shown to have greater germination at low water activity and greater pathogenicity to larvae of *Galleria mellonella* (Hallsworth and Magan, 1994b). However, the relative humidities at which the pathogenicity studies were conducted (86.5 and 100%) were very high. The results from the present study reflect these findings. It is likely that conidia produced by the 'dry' and 'washed' methods would have had an increased concentration of polyols, although this was not measured in this study. This may have improved the pathogenicity of these conidia, although a high relative humidity was still required for optimal mortality. This study has demonstrated that the production method can have an affect on the level of mortality produced by the conidia.

Conclusions

- The mass production treatment with no water produced significantly less conidia than those produced using 600 ml water/kg of rice.
- The degree of water stress in production did not significantly affect the ability of conidia to germinate over the range of water activities tested.
- The degree of water stress in production did significantly affect the initial viability of conidia for isolate IMI 389521 across treatments, but not for IMI 386243.
- In the bioassays, when initial humidity was high, the production method did affect the level of mortality produced by the conidia.
- The production method did not affect the ability of the conidia to cause mortality at a lower relative humidity.
- Isolate IMI 389521 performed better than isolate IMI 386243 in terms of viability and ability to germinate at lower water activities.

3.2.2. Step 1.2 Factors affecting insect susceptibility

Introduction

Several studies have shown the potential for entomopathogens to control a range of stored product insects either alone (Searle and Doberski, 1984; Adane *et al.*, 1996; Hidalgo *et al.*, 1998; Moino *et al.*, 1998; Rice and Cogburn, 1999; Meikle *et al.*, 2001, Sheeba *et al.*, 2001; Dal Bello *et al.*, 2001, Cherry *et al.*, 2005) or in combination with other control treatments such as diatomaceous earth (Lord, 2001; Akbar *et al.*, 2004; Athanassiou, 2004; Smith *et al.*, 2006; Vassilakos *et al.*, 2006; Athanassiou *et al.*, 2008). Much of this work has used species and conditions more likely encountered in tropical regions. Recent research has identified the most effective naturally-occurring insect-specific fungi in the UK for the potential application to structures for the control of residual infestations of storage pests (Cox *et al.*, 2004). This study also indicated that different beetle species had different susceptibilities to the fungus *Beauveria bassiana* (Balsamo) Vuillemin (Cox *et al.*, 2004). This work was done using a single concentration of conidia (1×10^8 conidia/ml) and it was not known whether the observed effect was due to innate differences in susceptibility between species or was an effect of the concentration used. i.e. would a higher concentration result in a high mortality for the less affected species.

Infection of an insect by entomopathogenic fungi occurs by a series of events. Some of the processes are known and understood, but there are many areas that still require clarification or investigation. The process can be divided into three parts: adhesion of the fungal conidia, penetration through the cuticle and establishment within the host. Much of the work examining adherence and penetration of entomopathogenic fungal conidia has been carried out using *Metarhizium anisopliae* (Metschnikoff) (see reviews of fungal pathogens by, for example St Leger, 1993; Hajek and St Leger, 1994), but some studies have also been made with *B. bassiana* (Pekrul

and Gula, 1979; Neves and Alves, 2004). Attachment of several species of entomopathogenic fungi to insect cuticle has been found to be passive and non-specific (Boucias *et al.*, 1988). It has been shown that the dry conidia of both *M. anisopliae* and *B. bassiana* are hydrophobic and suggested that hydrophobic interactions are responsible for adherence of the conidia (Boucias *et al.*, 1988; Jeffs *et al.*, 1999). Once the conidia have adhered to the cuticle and, in response to stimuli, the conidia will germinate and may eventually penetrate the cuticle as a result of both mechanical force and enzymatic degradation (Charnley and St Leger, 1991). Of the various processes that may be involved in determining virulence of an isolate one of the easiest to observe is adherence and germination of the conidia on the insect cuticle. In this study, we have established that there is a difference in susceptibility between species of stored product beetle and considered a possible explanation for this by examining the adherence and germination of *B. bassiana* using scanning electron microscopy. The possible influence of insect secretions was also investigated.

Materials and methods

Preparation of insects

a) Assessment of differences in susceptibility of species

The effect of *B. bassiana* IMI 386243 and IMI 389521 on adult *O. surinamensis*, *S. granarius* and *T. confusum* were compared. Insects were batched in groups of 20 in 7.5 x 2.5 cm glass tubes. Each batch of insects was treated individually by tipping the insects on to a glass Petri dish containing the dry conidia powder of the appropriate isolate. Individual insects were gently rolled over using fine forceps to ensure fungal conidia adhered to all parts of the insect exoskeleton. Control insects were treated in the same way but on a Petri dish without the conidia powder. The insects were then transferred to 9 cm diameter Petri dishes containing a filter paper moistened with 750 µl sterile distilled water, sealed with 'Parafilm' and kept at 20°C, 70% r.h. After 24 hours the insects were transferred to clean Petri dishes with a small amount of the appropriate foodstuff. Mortality was assessed 14 days after treatment. Dead insects were removed at the assessment period and surface sterilised by washing in 5% sodium hypochlorite for 5 seconds followed by three rinses in sterile distilled water. The cadavers were then placed in Petri dishes on filter papers moistened with sterile distilled water; after 5 days at 20°C, 70% r.h. they were examined for external sporulation of fungus to confirm that death was due to mycosis.

In a separate experiment, the effect of *B. bassiana* IMI 389521 on two strains of *T. confusum* and four strains of *T. castaneum* was examined to determine whether susceptibility was strain-dependant. The strains used were *T. confusum* W44, *T. confusum* susceptible, *T. castaneum* CTC12, *T. castaneum* lab., *T. castaneum* Bt1vs and *T. castaneum* FSSII. The insects were treated as described above and mortality was assessed after 14 days.

b) Assessment of conidial attachment and germination

A suspension of conidia of isolate IMI 389521 at a concentration of 1×10^8 viable conidia/ml suspended in sterile distilled water containing 0.02% Tween 80 was used to treat the insects. Suspensions were sonicated and vortex-mixed to ensure an even distribution of the conidia prior to use. Insects were batched in groups of 20 in 7.5 x 2.5 cm glass tubes. Each batch of insects was treated individually by transferring the insects into a 1.5 ml microcentrifuge tube and adding 1 ml of the conidial suspension. The tube was inverted slowly 10 times and the total length of immersion was approximately 30 seconds. The contents of the tube were poured into a Büchner funnel containing a Whatman No. 1 filter paper and the suspension was removed under vacuum. Each replicate treatment was transferred to an individual 9 cm diameter Petri dishes containing a filter paper moistened with 750 µl sterile distilled water, sealed with 'Parafilm' and kept at 20°C, 70% r.h. Control insects were treated with sterile distilled water containing 0.02% Tween 80. Insects were removed 24, 48 and 72 h after treatment for examination using scanning electron microscopy (SEM).

Examination of conidial attachment and germination using electron microscopy

a) Transmission electron microscopy

This work was carried out by the Bioimaging Core Technology Unit, Queens University, Belfast. Insects were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer and subsequently postfixed in aqueous 1% osmium tetroxide for two hours. Insects were washed in sodium cacodylate buffer overnight and then dehydrated through a series of ascending alcohols with two changes of 15 minutes each, ending with propylene oxide. Samples were then placed in a mixture of resin:propylene in the ratio of 1:3 for 2 hours, 1:1 for 2 hours and finally 3:1 for 1 hour before being placed on a rotator overnight. The following day samples were immersed in 100% resin for four hours and then embedded in rubber moulds for 48 hours at 60°C. Resin blocks were trimmed into a pyramidal shape with a block face size of approximately 0.25 mm square. Sections (60-90 nm) were cut using a diamond knife on a Reichert Ultracut E ultramicrotome. In a modification of this preparation method, the heads and the last 1 mm of the abdomen were removed from the insects prior to postfixing in osmium tetroxide to permit better permeation of the resin. Sections were stained in a saturated solution of uranyl acetate in 70 % ethanol for 10 minutes then in lead citrate stain for 8 minutes. Sections were viewed using a Phillips CM100 transmission electron microscope.

b) Scanning electron microscopy

Adherence and germination of fungal conidia was examined by low temperature scanning electron microscopy (cryoSEM). A Philips XL20 SEM with an Oxford Instruments CT1500 cryo system was used. The insects were positioned on an adhesive carbon disc and the samples were sublimated prior to sputter coating with gold/palladium. For each insect, an assessment of the number of

conidia present on three different areas of the body was made. These areas were the ventral abdomen, the hind femur and antennal segments 4-9. The total number of conidia present was recorded for the femur and antennal segments. For the ventral abdomen, twenty areas approx. 93 x 74 μm were chosen at random and the number of conidia within these areas were assessed. Thus, the area of the ventral abdomen examined for the two species was the same but for the hind femur and the antennal segments a larger area was examined for *T. confusum* as this is a larger species than *O. surinamensis*. Between 5 and 10 individuals of each species were examined at each of the three time periods post-treatment.

Involvement of cuticular components

To determine whether cuticular components or insect secretions could have a role in the tolerance of *T. confusum* to infection, SDA plates were inoculated with IMI 389521 and five individual insects of either *O. surinamensis* or *T. confusum* were placed on the surface of the agar of each Petri dish. Insects were killed prior to use by placing in a freezer for one hour. The plates were incubated at 25°C for 4 days and fungal growth was examined.

Further studies were made to examine possible causes for the observed zone of inhibition (see results). *Tribolium confusum* adults were placed in water or pentane (1 ml) in batches of 40 for one hour to extract water soluble or solvent soluble compounds. The ability of the resulting extracts to inhibit fungal growth was examined by removing small plugs of agar from SDA plates previously inoculated with IMI 389521 and pipetting 50 μl of the insect extract into the resulting well. Five wells were formed on each plate. Plates were incubated at 25°C for 4 days and the area of any observed zone of inhibition was measured

Effect of benzoquinones on fungal growth

One difference between the cuticular components of *Tribolium* spp. and *Oryzaephilus* spp. is the presence of benzoquinones, which act as a defensive secretion for *Tribolium* spp. The effect of benzoquinones on the growth of *B. bassiana* was, therefore, examined. A solution (50 μl) of methyl-1,4-benzoquinone (Aldrich Ltd, UK) (200 $\mu\text{g/ml}$) was placed in a well formed by removal of a plug of agar on an SDA plate inoculated with *B. bassiana* IMI 389521. Five wells were formed on each plate. Plates were incubated at 25°C for 4 days and the area of any observed zone of inhibition was measured.

Analysis of benzoquinones in *Tribolium* species

The amount of benzoquinones present in strains of *T. confusum* and *T. castaneum* was quantified using gas chromatography-mass spectrometry (GC-MS). Forty adult beetles of each strain were extracted in 1 ml of pentane for 1 hour. Aliquots (1 μl) of the solvent extracts were analysed on a Hewlett Packard 5890 series II gas chromatograph coupled to a VG Trio-1 mass spectrometer.

The extract was injected at 280°C in splitless injection mode (purge on after 1 min), onto a CPSil 5CB column (100% dimethylpolysiloxane, 50 m x 0.32 mm internal diameter, 1.2 µm film thickness). The oven temperature was initially 50°C for 1 min rising at 10°C per min to 310°C and held at this temperature for 30 min. Helium was used as the carrier gas, and the GC operated in constant pressure mode (5 psi, 31.3cm/sec at 50°C GC oven temperature, split vent flow 50 ml/min). The mass spectrometer source and interface temperatures were 200°C and 310°C respectively. The mass spectrometer was operated in electron impact mode (EI+) at 70 eV, and scanned from 33-550 amu once every second. Mass spectrometer acquisition commenced after 6 min (solvent delay time). A solution of methylbenzoquinone was used as an external standard to quantify the amounts of benzoquinones present in the solvent extracts.

Results

The pathogenicity of two different isolates of *B. bassiana*, IMI 386243 and IMI 389521 was examined with three different insect species using a high challenge test in which a large amount of conidia were in contact with the surface of the insect. High levels of mortality were recorded for *O. surinamensis*, but much lower mortality levels were found for *S. granarius* and these were lower again for *T. confusum* (Figure 5). Control mortality was less than 5% for all three species. For each individual species there was little difference between the efficacy of the two different *B. bassiana* isolates.

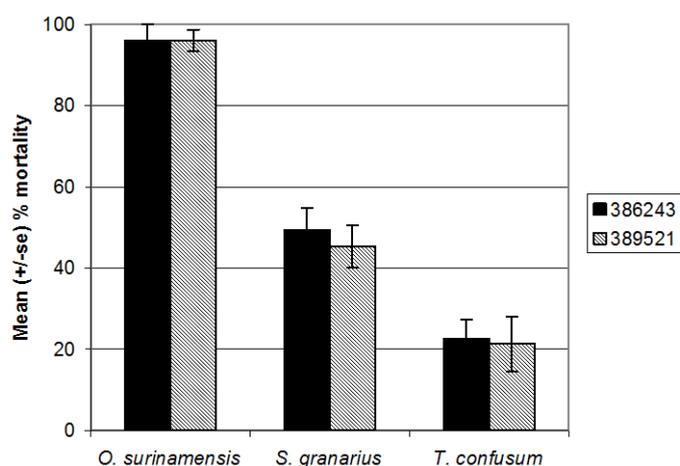


Figure 5. Mean % mortality (\pm SE) of three species of storage beetle 14 days after treatment with two isolates of *B. bassiana* dry conidia powder

One possible cause for the differences observed could be related to the adherence and germination of the conidia on the insect cuticle. This was examined using transmission and scanning electron microscopy (TEM and SEM). Three different body areas of *O. surinamensis* and *T. confusum* were examined by scanning electron microscopy after treatment with a conidial suspension. It was found that there were differences between the two species in terms of both the

number of conidia found and the number of germinating conidia seen (Figure 6 and Figure 7a-d). Examination at all three time periods post-treatment showed that conidia were present in greater numbers on the cuticle of *O. surinamensis* in all three body regions examined compared to the same regions for *T. confusum*. Observations at 24, 48 and 72 h post-treatment showed that some conidia on the cuticle of *O. surinamensis* had germinated, but this was observed for less than 5% of the conidia present.

Germinating conidia were not observed on the cuticle of *T. confusum* until 48 h post-treatment. The germ tubes seen were much shorter than those observed on *O. surinamensis* and fewer conidia were observed to show any sign of germination. It was found that for both species the number of conidia decreased with increasing time post-treatment.

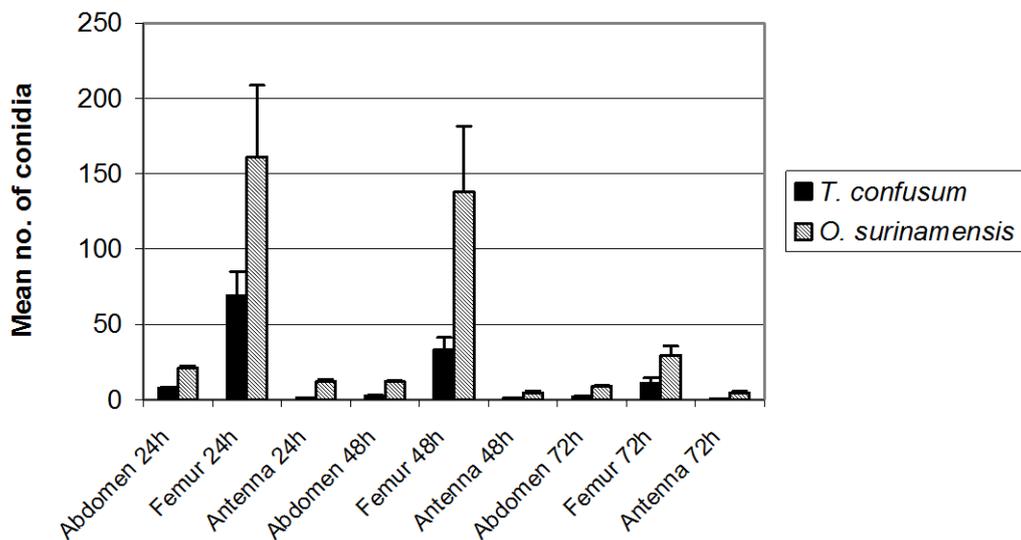
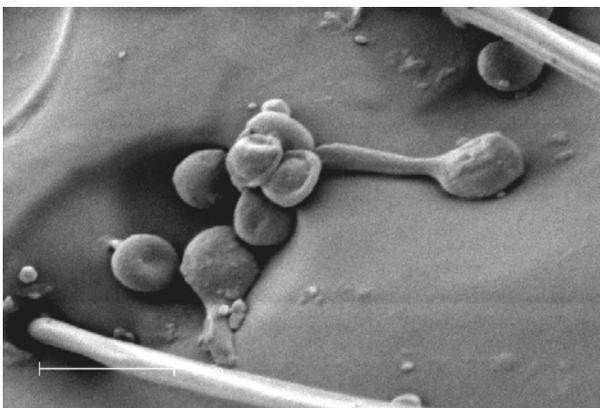
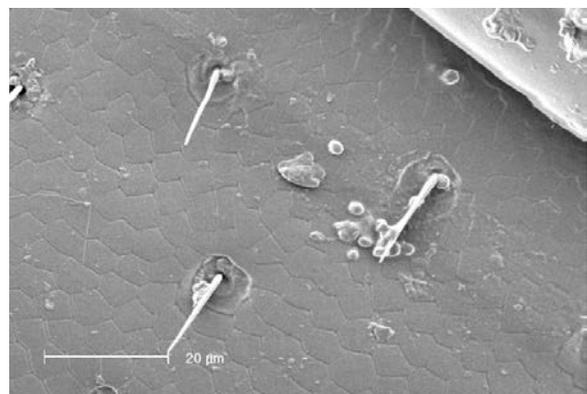


Figure 6. Examination of conidial attachment at different time periods post-treatment.

A. *O. surinamensis* 24 hr



B. *T. confusum* 24 hr



C. *O. surinamensis* 72 hr

D. *T. confusum* 72 hr

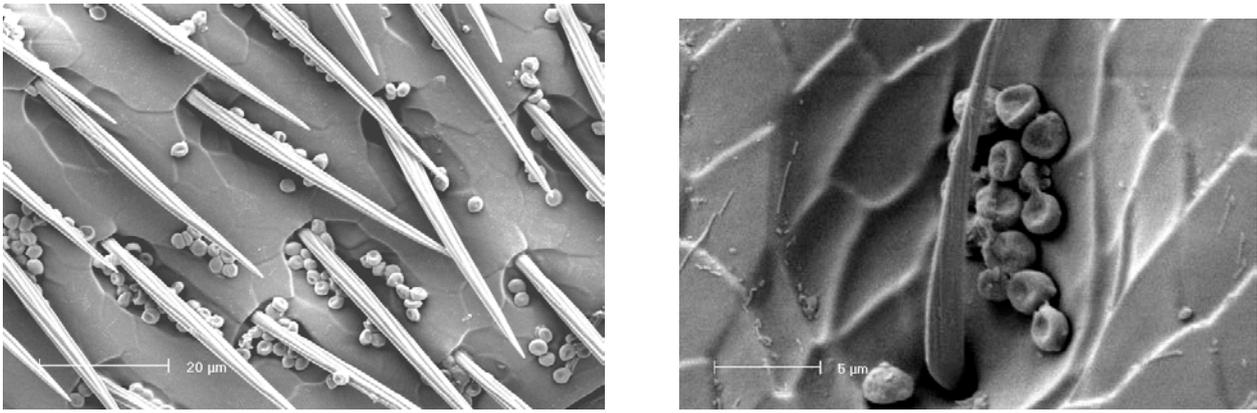


Figure 7. Attachment and germination of conidia of IMI 389521 on the ventral abdomen of *O. surinamensis* and *T. confusum*.

Examination of sections by transmission electron microscopy showed that fixation appeared good but resin infiltration was poor. Modification of the preparation method prior to postfixation gave slightly better results for *T. confusum* tissue but not for the *O. surinamensis* tissue. Penetration of the fungal hyphae through the insect cuticle was not observed for any of the sections photographed.

It was observed that the growth of IMI 389521 on agar plates on which individual adult *O. surinamensis* or *T. confusum* had been placed was inhibited in the proximity of *T. confusum* (Figure 8). Complete coverage was observed for plates that contained *O. surinamensis*, with the fungus covering the beetles (Figure 8). To investigate this further, solvent extracts of *T. confusum* were made and the effect of these on the growth of the fungus was determined. It was found that the extracts in water did not inhibit the growth of the fungus. In contrast, the insect extracts in pentane did inhibit fungal growth around the wells (Figure 9).

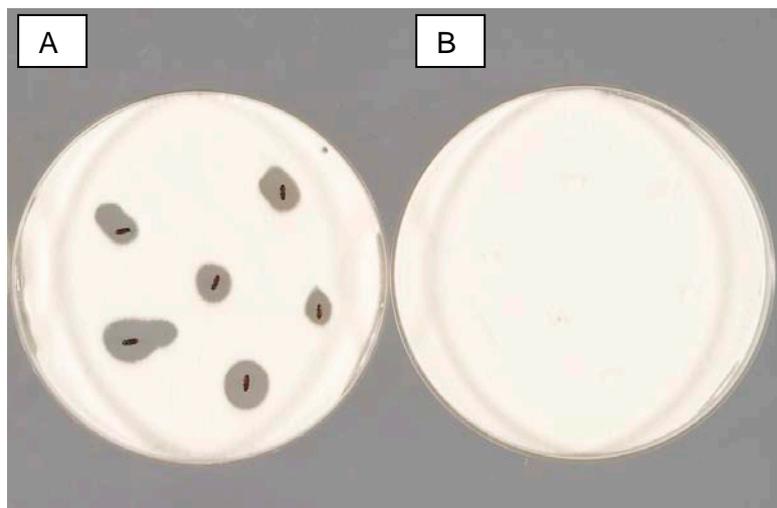


Figure 8. Growth of IMI 389521 on agar plates on which individuals of A. *T. confusum* or B. *O. surinamensis* were placed. Note the lack of growth around the adult *T. confusum*.

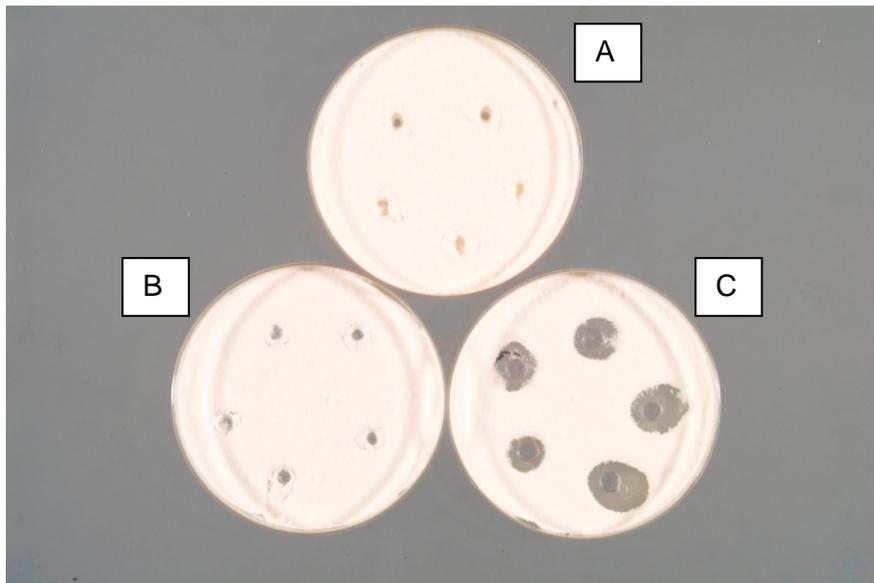


Figure 9. Growth of IMI 389521 on agar plates with wells containing A. pentane B. *O. surinamensis* extract in pentane or C *T. confusum* extract in pentane.

T. confusum is known to produce defensive secretions containing benzoquinones. The potential of benzoquinones to inhibit fungal growth was investigated for two strains of *T. confusum* and four strains of *T. castaneum*. Insect extracts in pentane from all six strains of *Tribolium* spp. resulted in the formation of zones of inhibition on SDA plates (Figure 10).

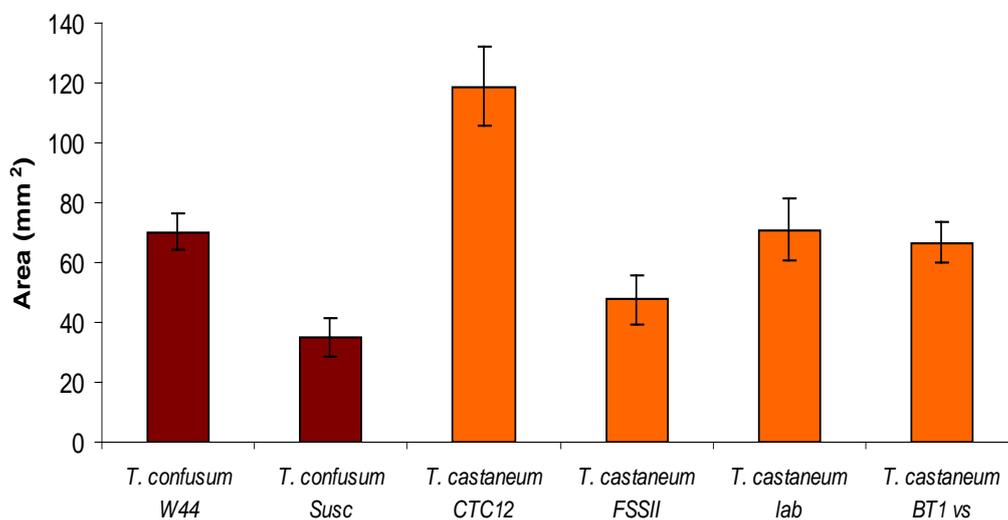


Figure 10. Mean (\pm SE) area of inhibition of growth of *B. bassiana* IMI 389521 around wells containing solvent extracts of different *Tribolium* species and strains.

The quantity of benzoquinones produced by the different strains was highly variable and the susceptibility of the six *Tribolium* strains to isolate IMI 389521 was also found to vary (Table 2). For some strains, for example *T. castaneum* CTC 12, the high level of benzoquinones was associated with a large zone of inhibition and low insect mortality.

Table 2. Levels of methyl- and ethyl benzoquinones found in six strains of *Tribolium* spp. and susceptibility to *Beauveria bassiana* IMI 389521. Figures show mean and standard error (N = 3).

Species and strain	Methyl benzoquinone ($\mu\text{g}/\text{beetle}$)	Ethyl benzoquinone ($\mu\text{g}/\text{beetle}$)	% Mortality after 14 days
<i>Tribolium confusum</i> W44	3.3 \pm 0.2	11.1 \pm 1.0	28.3 \pm 4.4
<i>Tribolium confusum</i> susc	6.5 \pm 0.6	18.2 \pm 1.9	11.7 \pm 1.7
<i>Tribolium castaneum</i> CTC12	13.5 \pm 1.3	23.9 \pm 2.7	13.3 \pm 6.0
<i>Tribolium castaneum</i> FSSII	4.5 \pm 0.3	7.3 \pm 0.9	20.0 \pm 5.8
<i>Tribolium castaneum</i> Lab	4.9 \pm 0.4	8.2 \pm 0.8	57.7 \pm 4.0
<i>Tribolium castaneum</i> Bt1vs	8.0 \pm 0.6	14.6 \pm 1.4	25.0 \pm 2.9

Discussion

The 'high challenge' test showed that the differences in storage beetle susceptibility to *B. bassiana* could not be explained as a function of the concentration of conidia used. The results confirmed those from a previous study (Cox *et al.*, 2004) that had used a concentration of 1×10^8 conidia/ml. An electron microscope study was undertaken to determine whether differences in adherence and germination of the conidia on the cuticle of *O. surinamensis* and *T. confusum* could explain the apparent differences. This study showed that quantitative and qualitative differences could be observed between the two species. *O. surinamensis* had a greater number of conidia adhering to the cuticle at each of the post-treatment periods. This species had a much greater number of setae, particularly on the ventral abdomen, and the presence of these may have assisted with adherence of the conidia. Germinating conidia were observed more frequently on the cuticle of *O. surinamensis* than for *T. confusum*. There are several possible reasons for this 1) the setae of *O. surinamensis* may trap air near the surface of the cuticle which, as a result of cuticular and respiratory transpiration processes, may contain higher levels of moisture. This would provide more favourable conditions for germination of the conidia. 2) The cuticular lipid profiles will differ between the two species. It is possible that the cuticle of *T. confusum* contains a substance that is inhibitory for conidia germination. It has been shown that cuticular hydrocarbons can either promote or inhibit conidial germination (St Leger, 1991 and references therein). *Tribolium* species are also known to produce defensive quinones and it is possible that these chemicals may also inhibit germination. Inhibition of yeast and bacterial growth by the defensive secretions from *Tribolium* spp. has been shown (Prendeville and Stevens, 2002). This hypothesis was explored further.

The number of conidia found on both species decreased over time. This could be as a result of grooming activities. From this, it would appear that conidia that have not germinated and

penetrated the cuticle within the first 24-48 hours are unlikely to remain on the cuticle and play a role in the infection process.

Penetration through the cuticle was not easily observed using the SEM method. Even at points where penetration may be expected such as intersegmental regions penetration could not clearly be seen. The lack of significant germ tube growth on the cuticle of both species may indicate that penetration occurs directly below the point of attachment. Both *B. bassiana* and *M. anisopliae* have been shown to produce germ tubes that grow over the surface of the insect cuticle until they contact an area of relative weakness where penetration can easily be achieved (Pekrul and Grula, 1979; Butt et al, 1995). Penetration by the fungal conidia is one of the factors that has been linked to virulence of various isolates of *Paecilomyces fumosoroseus* (Altre and Vandenburg, 2001). This study has shown that the early stages of fungal infection may play a key role in the susceptibility of storage beetles to some isolates of *B. bassiana*. The possible mechanisms for this require further investigation. It may be possible to increase susceptibility through the formulation of the conidia. In particular, compounds that are known to act on the cuticle could be incorporated if not detrimental to the conidia.

Tribolium spp. are known to produce mixtures of benzoquinones as a defensive secretion. The secretions of *T. confusum* and *T. castaneum* differ; the secretion of *T. confusum* consists of ethyl-1,4-benzoquinone (EBQ) and methyl-1,4-benzoquinone (MBQ), whilst that of *T. castaneum* consists of EBQ, MBQ and small quantities of 2-methoxy-1,4-benzoquinone (Pappas and Morrison, 1995; Unruh et al., 1998; Yezerski et al., 2000). In addition to their role in defence against both microbes and predators, MBQ and EBQ have also been shown to act as attractants for *T. confusum* at intermediate concentrations (Verheggen et al., 2007). Levels of MBQ and EBQ have been shown to differ between the sexes and between different strains of *T. confusum* (Yezerski et al., 2000). *Tribolium confusum* and *T. castaneum* have been found to inhibit the growth of some yeast and bacterial species and this was attributed to the presence of the benzoquinones (Prendeville and Stevens, 2002). Reduced growth of yeasts and bacteria in the presence of methyl-1,4-benzoquinone has also been reported (Yezerski et al., 2007). In the current study, we have shown that adult *T. confusum* can inhibit the growth of *B. bassiana* and that this can be attributed to the presence of benzoquinones. Only MBQ is available to purchase and, therefore, assays to examine the effect of benzoquinones on inhibition of fungal growth used MBQ only. The contribution of EBQ, or any additive or synergistic effects of MBQ and EBQ could not, therefore, be determined. The amount of benzoquinones present in the different strains of *T. confusum* and *T. castaneum* used in these studies was found to be very variable. The mortality caused by *B. bassiana* IMI 389521 was also found to vary between the strains. In the case of the two strains of *T. confusum* tested, the level of benzoquinones detected had an inverse relationship with the level of mortality i.e. the higher the level of benzoquinones present the lower the observed

mortality. This may be expected if benzoquinones are largely responsible for inhibition of fungal growth. The strains of *T. castaneum* tested did not always exhibit this relationship, for example *T. castaneum* FSSII. This may have been a result of the different batches of insects that were used for the analysis of benzoquinones and for the pathogenicity test as it is known that the level of benzoquinones produced are dependant on many factors, for example beetle age, nutritional status, density etc. Alternatively, this may indicate that substances other than the benzoquinones are also involved in the inhibition of fungal growth. Further testing is needed to ascertain this.

Conclusions

- Some insect species have a reduced susceptibility to infection by *B. bassiana*
- This may be due to physical characteristics e.g. absence of setae that restricts adherence of the conidia
- The presence of substances such as benzoquinones can inhibit fungal growth and are likely to play a role in the reduced susceptibility
- Formulation may overcome some of these factors

3.2.3. Step 1.3 Development of formulations

Introduction

The formulation of a biopesticide is an important aspect in product development as it may enhance the infectivity of the fungal conidia and allow a product to be stored over prolonged periods. Jones and Burges (1998) stated that formulations have four functions by which they act to enhance the active organism. Formulations may stabilise the active organism and prolong their storage, allow the organism to be applied easily to the target environment, protect it from any exogenous harmful factors and, finally, formulation may enhance its activity. By using the correct formulation, a product's stability, efficacy and delivery may be improved and it has the potential to be used in a wider range of environmental conditions (Boyetchko *et al* 1999). Oil formulations can be advantageous to an entomopathogen such as *Beauveria bassiana* for a number of reasons. Jones and Burges (1998) stated that by formulating in oil, or oil based emulsions, it may overcome the necessity of high humidity for the conidia to germinate. By formulating in oil, Bateman *et al* (1993) showed that a mycoinsecticide can be more effective at lower humidities than the same conidia formulated in a water-based carrier. The conidia of *B. bassiana* are lipophilic and formulating in an oil formulation allows the conidia to mix easily when preparing the formulation. When formulating in water-based formulations, it is necessary to use a surfactant, for example Tween 80, that breaks the surface tension of the water and allows conidia to suspend. Additional formulating ingredients may also enhance the uptake of conidia onto the insect cuticle, for example the addition of electrostatic powders to dust formulations. Inert dusts such as clays or silica are often used in powder formulations and can bulk the formulation (Jones and Burgess 1998), enhance the activity of the agent and increase the persistence of conidia.

Storage life of a biopesticide is also an important aspect to take into account during product development, as this will affect the storage and distribution system of the final product. Wright *et al.* (2001) gave a brief overview of formulated shelf lives of various products and, in general, studies quoted have achieved over 12 months, either stored at cool or ambient temperatures.

The aims of this study were to assess the viability of conidia of isolates IMI 389521 and IMI 386243 over time in various formulating agents. A range of formulations were considered including both liquid and dust formulations, various bulking agents and liquid emulsions. In addition, the effect of the formulations on insect mortality was also assessed to determine the optimal formulation for use in further studies.

Materials and methods

Assessment of viability of isolates in different formulations

A variety of different formulations were prepared using *B. bassiana* isolates IMI 389521 and IMI 386243. An overview of the experiments carried out using the various isolates and groups of formulating agents is shown in Table 3.

Table 3. The length of time over which the viability of *Beauveria bassiana* IMI 389521 and IMI 386243 were assessed using various groups of formulating agents.

FORMULATION TYPE	STORAGE TIME (MONTHS)	
	IMI 389521	IMI 386243
Mineral oils	12	N/A
Powder	12	12
Vegetable oil	12	12
Emulsifier	12	12
Water-based	12	12

In each of the formulation experiments (Table 4), 0.1g of *B. bassiana* conidia (IMI 389521 or IMI 386243) were weighed out into clean 30 ml glass vials. Formulating agents were added and vials were sonicated for 3 min prior to incubation in order to break up aggregations of conidia. Two sets of formulations were prepared in each experiment, of which one set was stored at 5°C and the others were stored at 25°C. Regular germination tests were carried out on the formulations in order to assess their viability over time. For each formulation experiment, 3 reps were carried out over time (unless indicated).

To assess germination a small amount of powder (approx 0.005g) or 1-2 drops of liquid formulations were added to 9ml of Shellsol T. Formulations 6 and 10 were added to distilled water, as they were water-based formulations. Suspensions were sonicated for 3 min in order to break up any aggregations of conidia, then 1-2 drops of each suspension were spread across 3 x Sabouraud Dextrose Agar plates using the back of a micro-spatula. Plates were incubated for 24 hrs at 25°C before being assessed for germination. Plates were read at x 200 magnification and at least 300 conidia were observed on each plate. Conidia were considered to be germinating when the length of the germ tube exceeded the diameter of the conidia. Germination was considered to be excellent if over 90% and good if over 70%.

Table 4. An overview of the formulations tested showing the formulating ingredients, the isolate used and the duration of the experiment.

Formulation number	1 st formulating ingredient	2 nd formulating ingredient	Storage Time (months)	No. reps 389521	No. Reps 386243	
					Batch 018	Batch 019
1	10 ml Shellsol T	-	12	3		
2	10 ml Ondina EL	-	12	3		
3	5 ml Shellsol T	5 ml Ondina EL	12	3		
4	0.005g Entostat (5%)	-	12	3	3	3
5	0.005g Entostat (5%)	Globrite 10%*	12	3	3	3
6	1 ml Codacide ⁺	9 ml tap water	12	3		
7	0.005g Entostat	0.1g Gasil 23D (bulking agent)	12	3	3	3
8	0.005g Entostat+10% globrite	0.1g Gasil 23D (bulking agent)	12	3	3	3
9	10ml 0.05% Tween 80	-	12	3	3	3
10	10 ml Codacide ⁺	-	12	3	3	3
11	10 ml Olive oil	-	12	2		2
12	10 ml Corn oil	-	12	2		2
13	10 ml Sunflower oil	-	12	2		2
14	10 ml Rapeseed oil	-	12	2		2
15	10 ml Light White Mineral oil	-	16	3		2
16	Diatomaceous earth	-	11	1		

17	Cornflour	-	11	1		
18	Talc	-	11	1		

The effect of formulated conidia on *Oryzaephilus surinamensis*

The oil and dust formulations (1-15) described in Table 4 were tested against the saw-toothed grain beetle (*O. surinamensis* (strain Tram)). In addition, silicone oil was also tested. Oil and water-based formulations were applied using a potter tower (Cox *et al.*, 2004). The oil or water-based formulation (1 ml) was sprayed on to a Petri dish (90 mm diameter) containing 20 adult *O. surinamensis*. Dry powder formulations were applied by mixing an appropriate quantity of the formulation with 0.5 g of rolled oats in a 90 mm diameter Petri dish. Three different concentrations of conidia were tested for each formulation. Control treatments consisted of the formulation ingredients without the conidia. As the quantity of the dry powder formulations differed dependant on the concentration of the conidia, the control treatment used the quantity of the formulation present in the highest conidia concentration. Adult *O. surinamensis* (20) were added to each dish. For all formulations tested, insects were held at 20°C, 70% r.h. and mortality was assessed after 14 days. There were five replicates for each treatment.

Results

Viability studies at 5°C and 25°C

Mineral-based oils (excluding light white mineral oil)

Beauveria bassiana IMI 389521 retained excellent viability over 365 days when stored in mineral-based oils (Figure 11). No significant difference was observed between the powder control of conidia only and the three mineral oil formulations, at both temperatures ($F=3.67$, $P>0.05$ at 5°C and $F=0.04$, $P>0.05$ at 25°C). At 25°C, good germination was retained until 301 days after which viability showed a decline. An analysis of variance showed that there was no significant difference between the viability at Day 1 and Day 365 at 5°C ($F=1.13$, $p>0.05$). IMI 386243 was not formulated in these oils.

Powder formulations

Initial germination for all formulations was excellent at over 90% at both temperatures for IMI 389521, and this level of viability was retained for 365 days at 5°C with no significant difference between viability at Day 1 and Day 365 ($F=0.68$, $P>0.05$; Figure 12). At 25°C, viability remained good until 301 days, but dropped to around 60% thereafter. No significant difference was seen between the formulated and control conidia after 365 days at both temperatures ($F=1.02$, $P>0.05$ at 5°C and $F=0.18$, $P>0.05$ at 25°C).

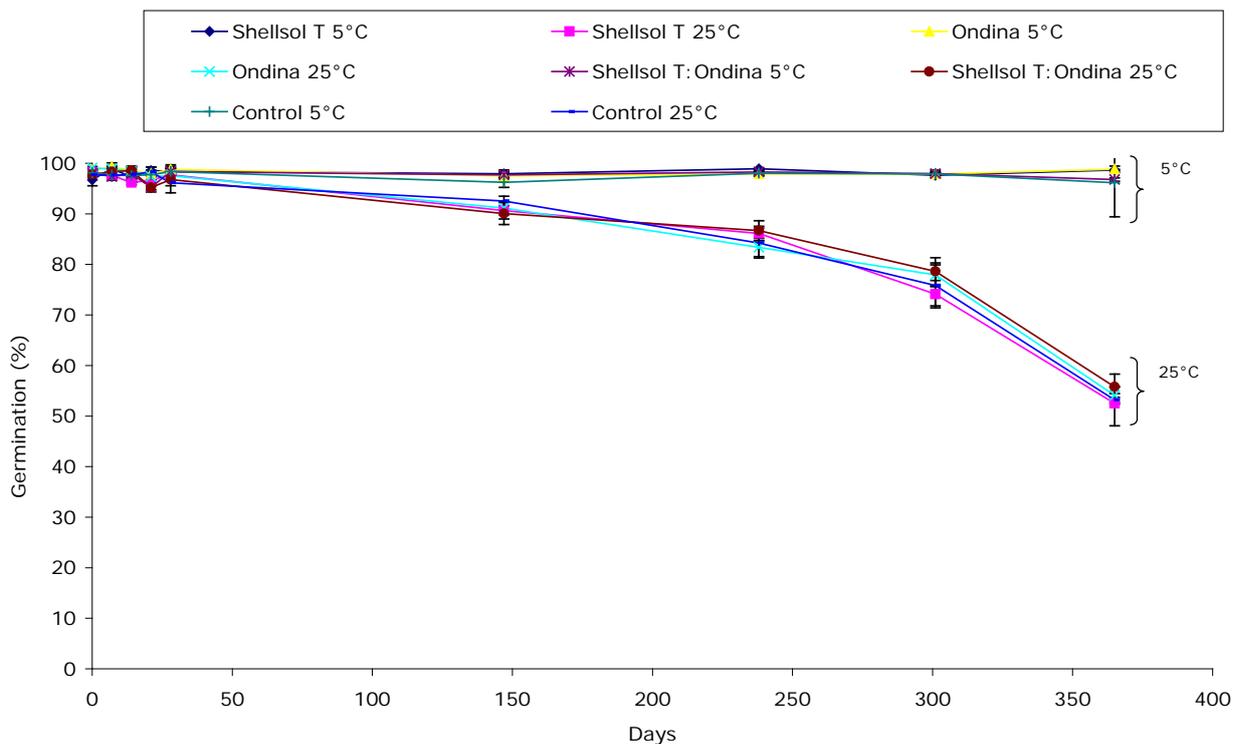


Figure 11. Viability (mean \pm 1 standard error (se)) of mineral oil formulations over 365 days prepared with isolate IMI 389521

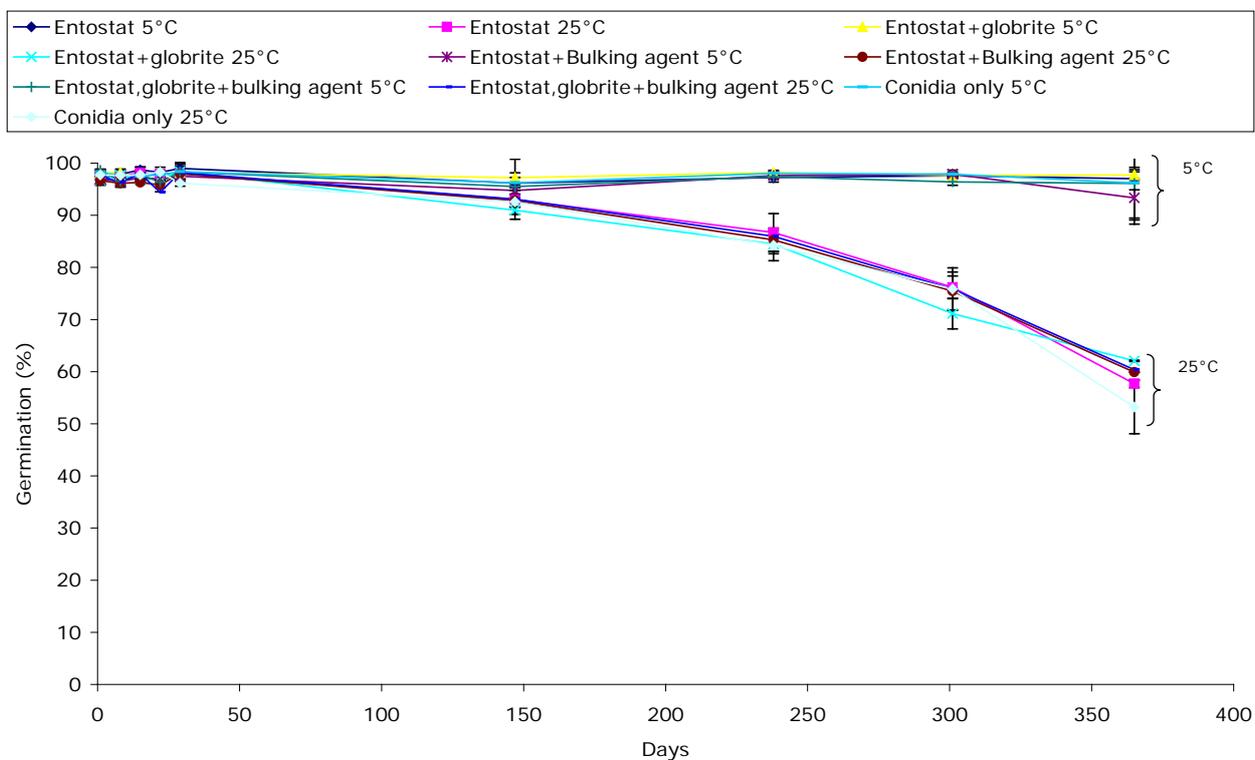


Figure 12. Viability (mean \pm SE) of powder formulations over 365 days prepared with isolate IMI 389521.

For IMI 386243, initial germination of all formulations was below 90%. At 5°C, there was no significant difference in germination between formulated conidia and the control after 365 days

($F=2.26$, $P>0.05$, Figure 13) and no significant difference between germination at Day 1 and Day 365 ($F=1.17$, $P>0.05$). Both the control and formulated conidia showed a decline in viability over 365 days. At 25°C, viability of all formulations was below 20% after 365 days. However, those formulated in the bulking agent Gasil 23D had significantly higher germination after 365 days than those not formulated in Gasil 23D and the control (Tukey HSD $P<0.01$)

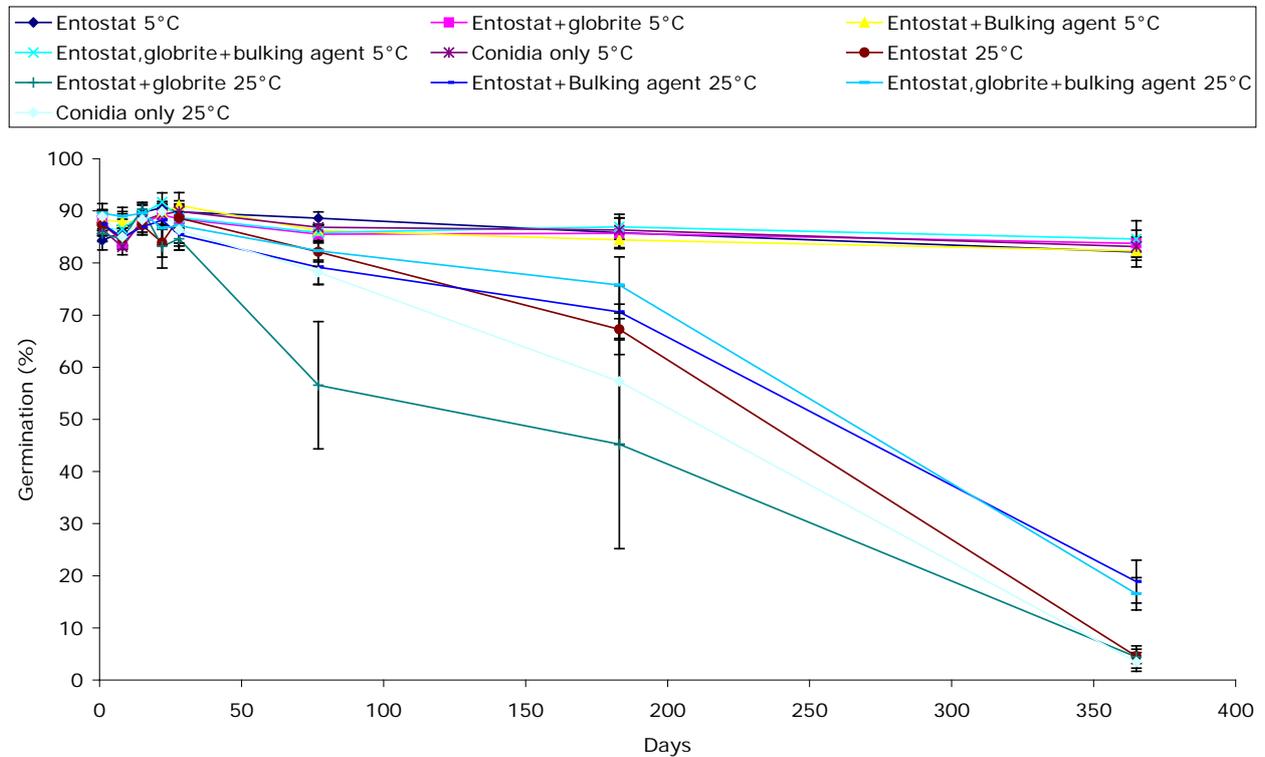


Figure 13. Viability (mean ± SE) of powder formulations over 12 months prepared with isolate IMI 386243 (batch 019).

Vegetable-based oils

When formulated in vegetable-based oils, the viability of conidia in general was excellent over 365 days at 5°C for IMI 389521 and good to excellent for IMI 386243 (Figures 14 and 15), apart from conidia formulated in the vegetable oil (which was rapeseed based). A decline in germination, was observed for many of the formulations stored at 25°C; especially for rapeseed based oils (this includes the ‘vegetable’ oil) for both isolates. The control for both isolates also declined at 25°C but, in general, it was only rapeseed oil and vegetable oil that declined more rapidly than the control.

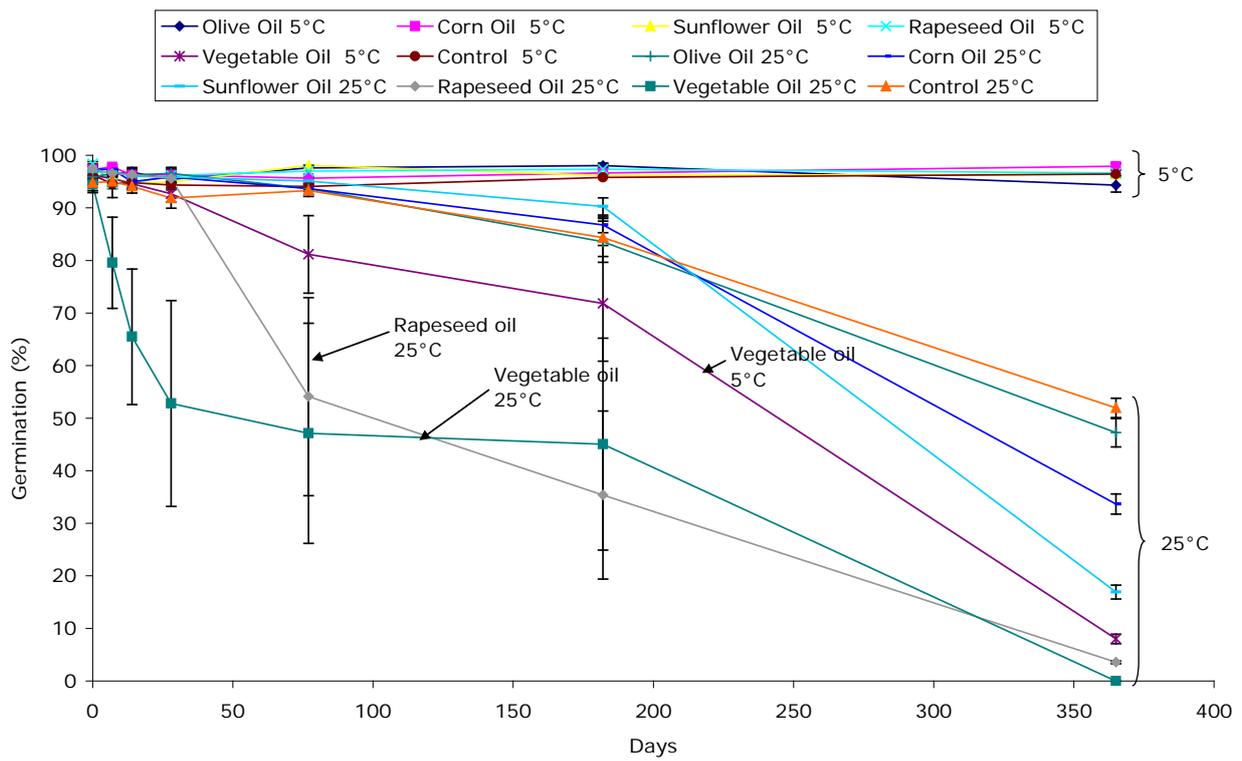


Figure 14. Viability (mean \pm SE) of IMI 389521 in vegetable-based formulations over 365 days. Note: the 365 day time point is only made up of 1 rep.

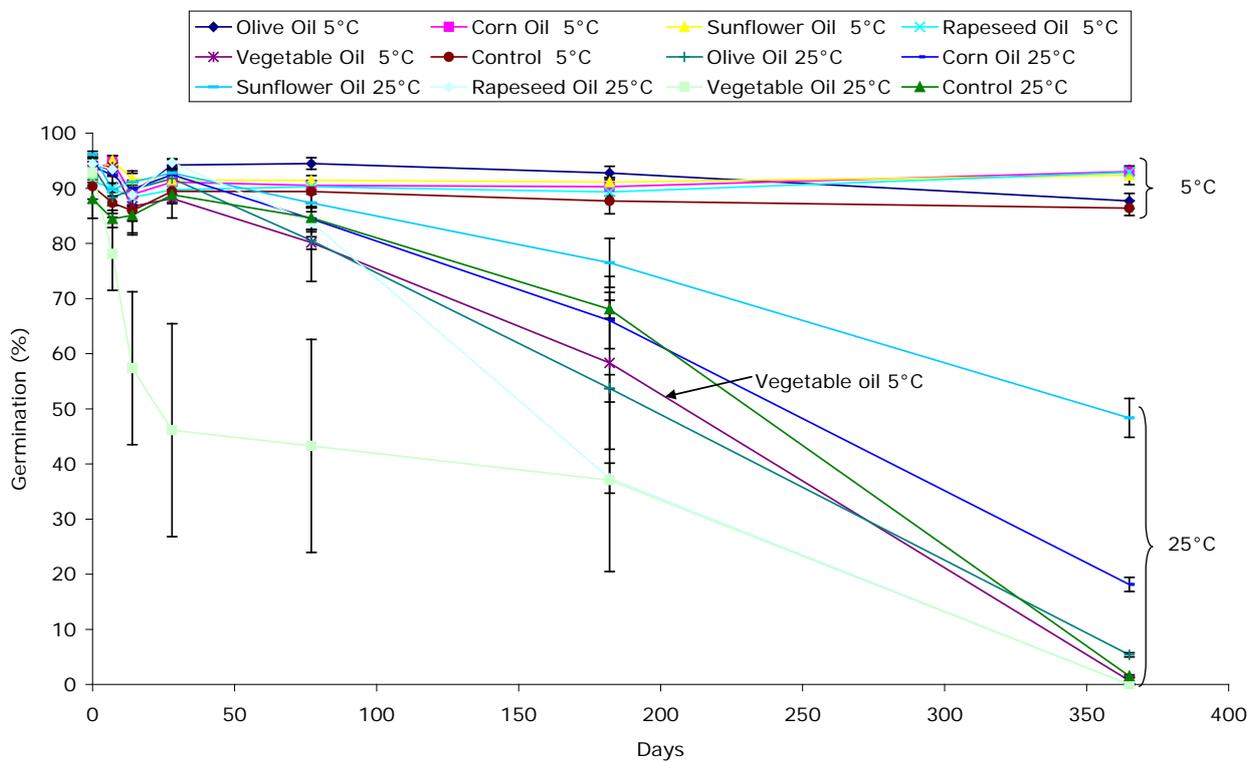


Figure 15. Viability (mean \pm SE) of IMI 386243 in vegetable-based formulations over 365 days. Note: the 365 day time point is only made up of 1 rep.

Emulsifier

Both isolates were formulated in neat codacide as described and two different batches of IMI 386243 were tested. IMI 389521 retained excellent viability over the 365-day assessment period at 5°C and good viability at 25°C dropping to around 80% after 365 days (Figure 16). It must be noted that the graph shows a jump in viability for isolate IMI 389521 near the beginning of the experiment, due to error (the nature of the formulating agents means that, when spread on agar, oil droplets are more difficult to separate from conidia). IMI 386243 showed a rapid drop in viability after 28 days at 5°C and 7 days at 25°C (Figure 16). The viability of the control for both batches at 25°C dropped much more slowly, reaching almost 0% after 365 days as opposed to 7 days when mixed with codacide. A similar pattern was shown for both batches of IMI 386243 at 5°C, although the control viability did not drop to zero (Figure 16).

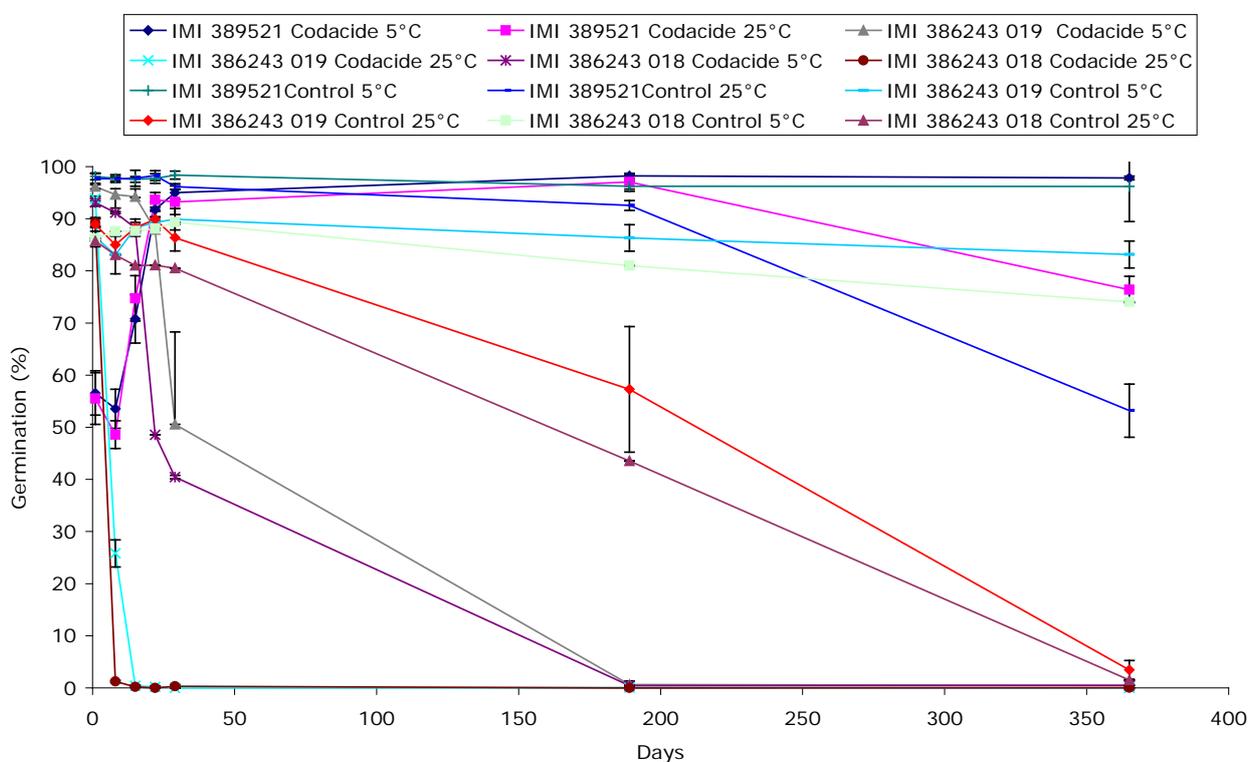


Figure 16. A comparison of viabilities of IMI 389521 and two batches of IMI 386243 (018 and 019 prepared by Sylvan) in neat emulsifier over a period of 365 days (mean \pm SE). NB the control for IMI 389521 was taken from a previous experiment as no control was run alongside these experiments.

Water-based formulations

The results for conidia formulated in a water based formulation (Tween 80, 0.05%) showed that viability dropped significantly when compared to the control for both isolates (Figures 17 and 18). No significant drop in viability was observed in the control viability at 5°C for either isolates ($P > 0.05$ IMI 389521, $P > 0.05$, IMI 386243 Tukey HSD). After 147 days at 5°C, viability of the conidia formulated in Tween 80 was significantly less than the control for IMI 389521 ($P < 0.05$) and after 183 days (no 147 day reading was taken) for IMI 386243 ($P < 0.01$). Conidia formulated in Tween

and incubated at 25°C showed a rapid decline in viability falling to 0% for both isolates, with viability significantly less than the control after 22 days for IMI 389521 ($P < 0.05$) and 7 days for isolate IMI 396243 ($P < 0.01$, Tukey HSD).

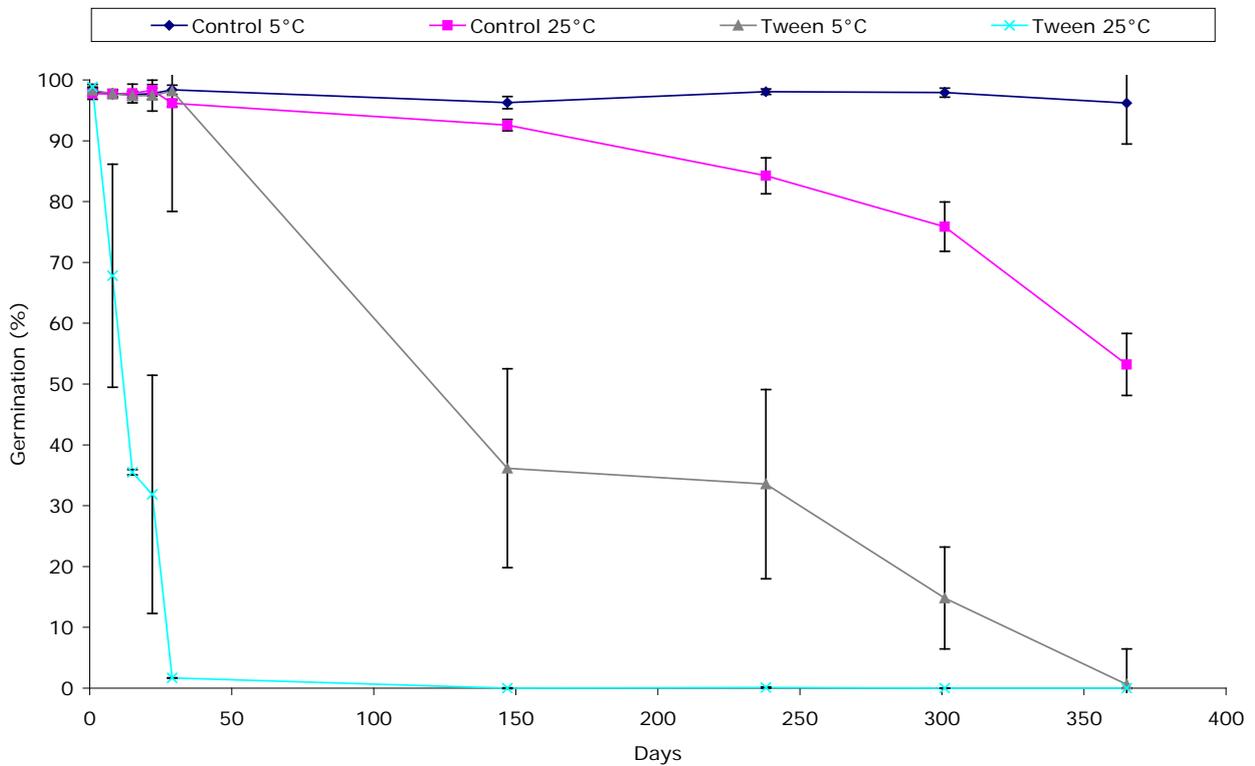


Figure 17. Viability (mean \pm SE) of conidia of IMI 389521 formulated in 0.05% Tween over 365 days.

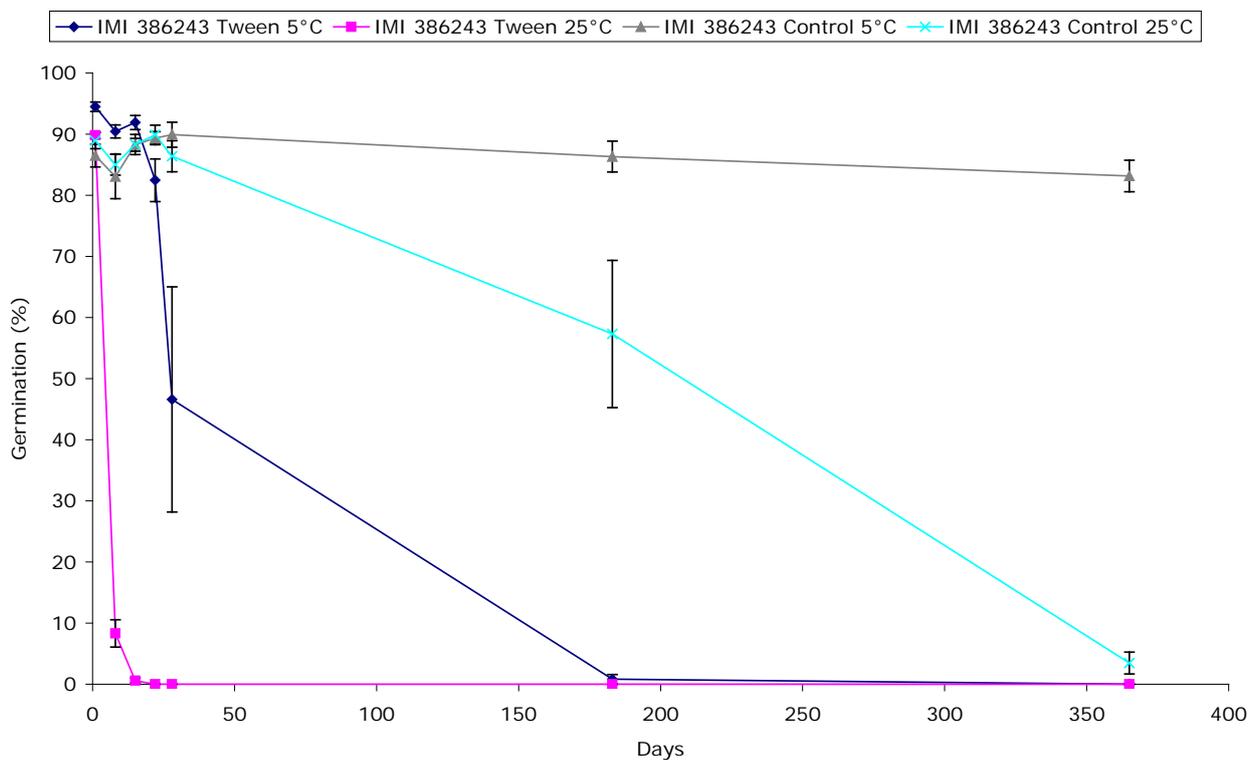


Figure 18. Viability (mean \pm SE) of conidia of IMI 386253 formulated in 0.05% Tween over 365 days.

Light white mineral oil

Viability of IMI 389521 formulated in light white mineral oil remained excellent over the period of 518 days with the final average formulated viability of 90.9% (\pm 1.93 %, Figure 19). There was no significant difference between the formulated and control conidia at 5°C after 365 days ($F=8.11$, $P>0.05$). Viability remained good at 25°C for 180 days with an average viability of 84.7%. After this point the viability dropped to almost zero, with the control having significantly higher germination than the formulated conidia ($F=33.27$, $P<0.01$). Little variation was observed between replicates for this isolate apart from replicate 3 on the final two readings. These data points were removed, as they were anomalous. Results for IMI 386243 were much more variable (Figure 20). Only two replicates were set up for this isolate, and viability declined at different rates in each replicate, causing the larger error bars observed in Figure 20. Viability, however, was similar to that of the control at each temperature; therefore, the decline was probably due to the isolate and not the formulating agent. Good germination was observed for IMI 386243 up until 180 days at 5°C.

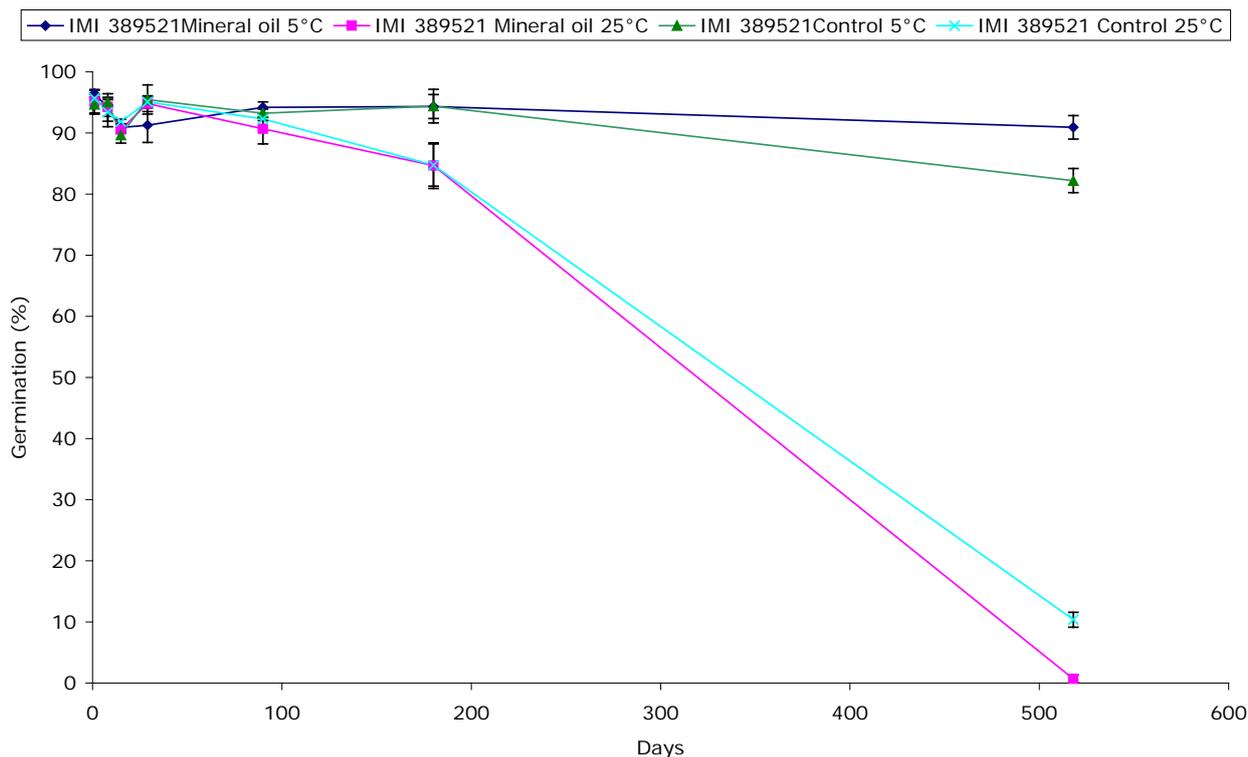


Figure 19. Viability (mean \pm SE) of IMI 389521 conidia formulated in light white mineral oil over 518 days. Three replicates were run, but results for mineral oil rep 3, 5°C on day 180 and 518 were removed due to anomalous results.

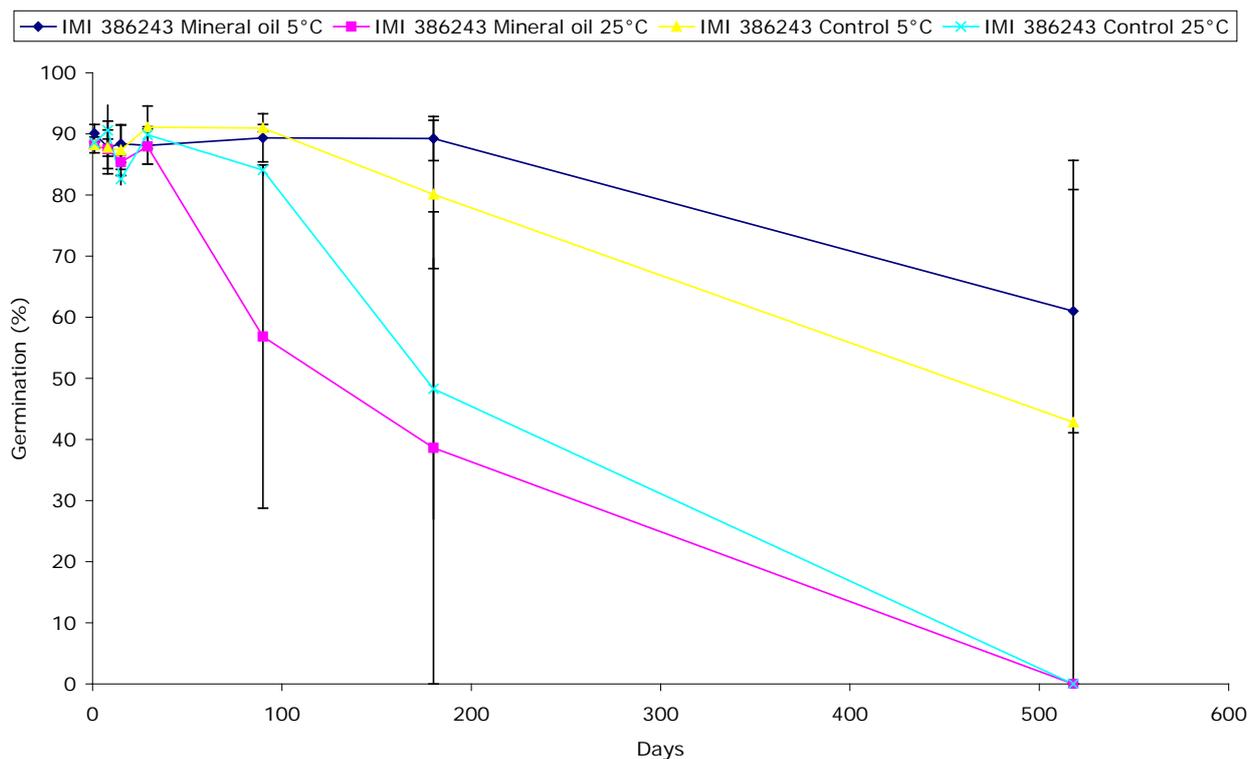


Figure 20. Viability (mean \pm SE) of IMI 386243 conidia formulated in light white mineral oil over 518 days.

Bulking agents

This study was a preliminary study investigating the effects of bulking agents on the viability of conidia over time; therefore, only one replicate was set up. Over the period of 322 days, good viability was maintained at 5°C with cornflour and diatomaceous earth (DE). However, conidia prepared with talc showed a drop in viability (Figure 21). Viability dropped to almost zero at 25°C for DE and cornflour formulations. More replicates would need to be carried out on bulking agents to confirm these results.

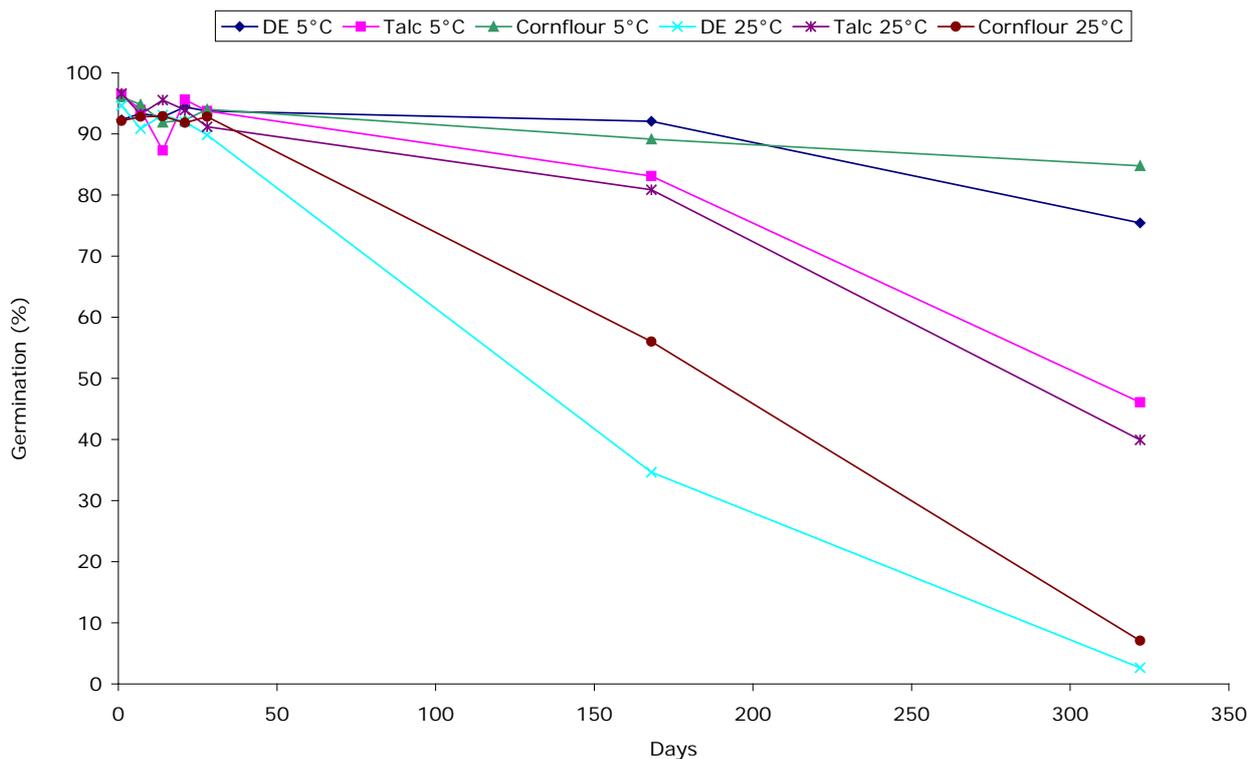


Figure 21. Viability of conidia prepared with various bulking agents over 322 days. Only 1 replicate was carried out.

The effect of different formulations on mortality of *O. surinamensis*

Vegetable oil-based formulations and a silicone oil-based formulation were compared with a water-based formulation containing 0.05% Tween 80. The concentration of *B. bassiana* IMI 389521 was 1×10^8 conidia/ml. Mortality of insects treated with the carrier oils or Tween 80 formulations containing IMI 389521 was always higher than mortality with carrier alone (Figure 22). However, the mortality with all treatments was generally very low. The silicone oil containing IMI 389521 gave the highest mortality, but mortality with the silicone oil alone was higher than usually found for a control treatment.

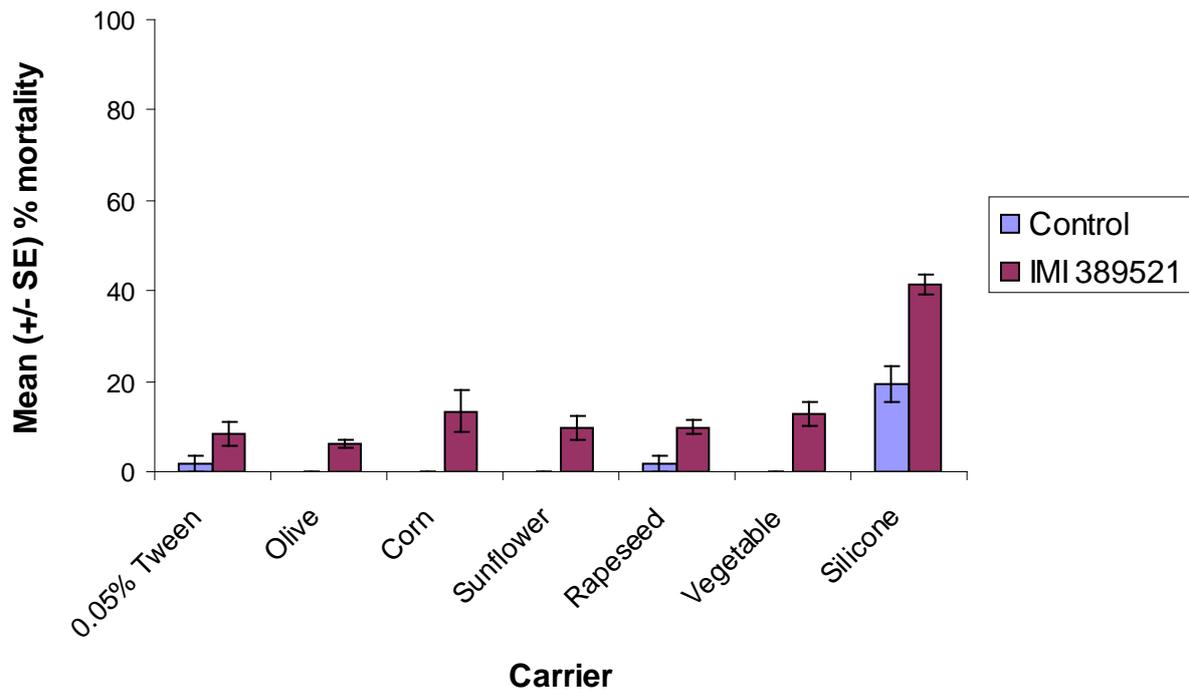


Figure 22. Mean (\pm SE) % mortality of *O. surinamensis* 14 days after treatment with *B. bassiana* IMI 389521 formulated in different carriers at a concentration of 1×10^8 conidia/ml.

Vegetable oil and silicone oil were selected for comparison with 0.05% Tween 80 at a higher concentration of conidia (1×10^9 conidia/ml). Mortality with the oil formulations was much greater than with the water-based Tween 80 formulation with this concentration of *B. bassiana* (Figure 23). However, it was found that at this increased concentration the nozzle of the Potter tower frequently blocked leading to inconsistent spraying. The other vegetable oils were, therefore, not compared at this concentration.

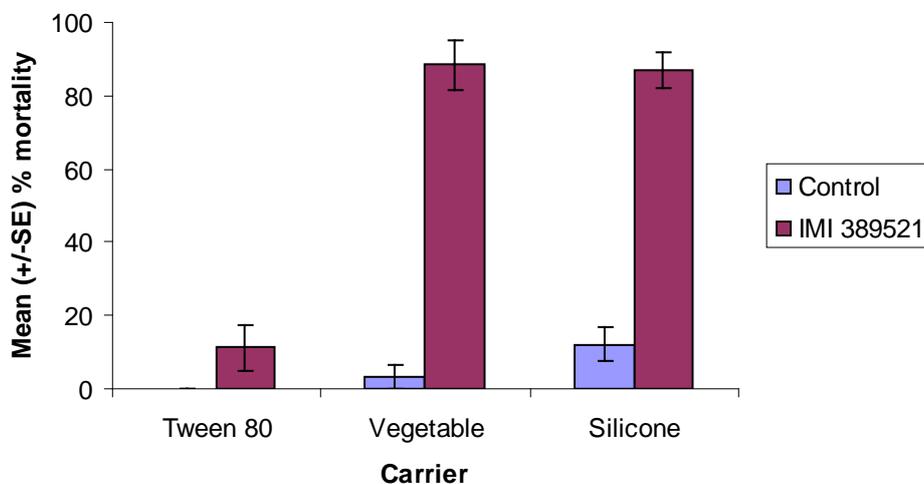


Figure 23. Mean (\pm SE) % mortality of *O. surinamensis* 14 days after treatment with *B. bassiana* IMI 389521 formulated in different carriers at a concentration of 1×10^9 conidia/ml.

Beauveria bassiana IMI 389521 at a concentration of 1×10^9 conidia in a powder formulation resulted in high mortality (91.3%) of *O. surinamensis* (Table 5). The addition of Entostat (5%) did not affect the level of mortality observed at any of the three concentrations compared with the conidia alone (Table 5). Similarly, the presence of Entostat and Globrite did not affect the levels of mortality observed. Higher levels of mortality were seen at the two lower concentrations for the formulations in which Gasil 23D was an ingredient. However, it will be noted that the control mortality for these formulations was nearly 100%, the same as was seen for the formulation containing 1×10^9 conidia (Table 5). As the quantity of the formulation ingredients was the same for the highest fungal concentration and the control, it can be concluded that Gasil 23D is causing a significant degree of insect mortality at this quantity. The mortality observed at the lower fungal concentrations is, therefore, likely to be due, in part, to the Gasil 23D component.

Tests to examine the effect of the mineral oils Shellsol T and ondina resulted in very high control mortality (>50%) when insects were sprayed using the Potter tower (results not shown). Other spray techniques were examined but it was not possible to reduce the control mortality under laboratory conditions. Tests of IMI 389521 in these carriers were not, therefore, possible.

Table 5. Mean (\pm SE) % mortality of *O. surinamensis* 14 days after treatment with *B. bassiana* IMI 389521 formulated in different powder based carriers at three different concentrations.

Formulation	Control	1×10^7 conidia	1×10^8 conidia	1×10^9 conidia
IMI 389521 dry powder	0 \pm 0	1.0 \pm 1.0	23.7 \pm 3.0	91.3 \pm 3.1
Entostat (5%)	0 \pm 0	1.0 \pm 1.0	37.0 \pm 5.1	82.7 \pm 6.1
Entostat (5%) & Gasil (100%)	99.1 \pm 1.0	42.4 \pm 4.0	38.4 \pm 3.0	100 \pm 0
Entostat (5%) & Globrite (10%)	0 \pm 0	11.2 \pm 2.0	29.7 \pm 3.4	86.4 \pm 2.9
Entostat (5%) & Globrite (10%) & Gasil (100%)	100 \pm 0	12.8 \pm 6.1	77.9 \pm 2.6	100 \pm 0

Discussion

The formulation studies have shown that in an appropriate formulation, at 5°C, the isolate retains excellent germination over a period of 365 days. The experiments using light white mineral oil showed that the germination remained over 90% in the formulated product for isolate IMI 389521 over 518 days. In general, IMI 389521 performed better than IMI 386243 with higher initial germination and more reproducible results in experiments. At 25°C, the experiments have shown that, in general, good germination may be achieved after 301 days of storage in mineral based oils and the powder formulations, with viability remaining above 70%. The higher viability achieved through storage at 5°C reflects findings by Jaronski (1997), whereby conidia of *B. bassiana* retained higher viability over one year, when compared to those at 25°C. Vegetable oils in general gave excellent results at 5°C. However, it was found that there were differences between oils

produced from the same type of crop plant. For instance, vegetable oil and rapeseed oil were both rapeseed oil based but very different results were obtained from isolates formulated in each. It could be hypothesised that there is more batch variability with vegetable-based oils and the presence of substances such as fungicides etc is a possibility but was not determined in these studies. There could also be a higher chance of rancidity with the use of vegetable oils. Jaronski (1997) reported that high temperature stability of *B. bassiana* conidia was better in paraffinic oils (petroleum based) when compared to vegetable oils at 25°C.

It is clear from the results that water-based formulations such as 0.05% Tween 80 were not suitable for either isolate as viability was lost very rapidly at 25°C and less rapidly at 5°C. There was also an apparent isolate difference when exposed to the emulsifier, Codacide. IMI 386243 lost viability rapidly at 25°C, whereas this effect was not seen with IMI 389521. Jaronski (1997) reported that a toxic effect on conidia stored in emulsifiers may be observed, and tested a number of different emulsifiers against *B. bassiana* strain GHA, showing that the isolates viability varied with the emulsifier used. We have shown here that the effect of one emulsifier has different effects on different isolates of *B. bassiana*.

The oil formulations at the higher concentration resulted in greater mortality of *O. surinamensis* in comparison with the water-based Tween 80 formulation. This reflects previous studies that have shown that oil-based formulations may cause higher mortality than water-based formulations (Bateman *et al.*, 1993). The powder-based formulations also showed potential. The addition of Gasil 23D as a bulking agent resulted in high insect mortality. Gasil 23D is a silica-based product and, therefore, could have a similar mode of action to that of diatomaceous earths. It is likely that the addition of Gasil 23D produced a desiccant effect on the insects resulting in the observed mortality. The addition of Entostat and Globrite did not affect insect mortality at the concentrations used and could, therefore, be included in a powder-based formulation.

Conclusions

- Isolate IMI 389521 showed better viability than IMI 386243 in most formulations.
- Powder formulations, the emulsifier and the mineral-based oils retained excellent germination at 5°C.
- Oil formulations had greater efficacy against *O. surinamensis* than a water-based formulation.
- Powder-based formulations also show good potential for control of *O. surinamensis*
- Based on the viability and efficacy results, oil- and powder-based formulations look to be good candidates for future use as commercial mycoinsecticide formulations.

3.3. Objective 2 To improve the delivery of the fungal conidia to the insects.

Optimising the efficacy of the putative agent when it is in contact with insects is necessary for success but not sufficient: it is essential to ensure that the right amount of the agent is brought quickly enough into contact with the target insects, which may be hiding in cracks and crevices, to ensure control of a pest population. Evidence that this requires further research was obtained by Cox *et al.* (2004), who showed that insect mortality was high only when insects were either rolled in conidia or were sprayed directly with them. The problem was addressed in three steps. **Step 2.1:** In principle, the initial uptake of conidia by insects can be achieved by either moving the insects to the conidia or *vice versa*. Both approaches were investigated to establish the most promising application methods to achieve initial infection of a pest population, whether freely wandering or hidden within the fabric of the store. **Step 2.2:** After initial infection of some insects in the infestation, the movement of those individuals can help to disperse the infection by two mechanisms. Both mechanisms were studied to establish how their contribution to control of pest populations can be maximised. **Step 2.3:** Based on the findings from objective 1 and steps 2.1 and 2.2 the most effective isolate was chosen. This step provided evidence that the chosen isolate can be produced on a sufficient scale to be commercially viable and confirmed the efficacy of the isolate produced by the mass production method in laboratory tests.

3.3.1. Step 2.1 Improving the uptake of fungal conidia

Moving the insects to the conidia

Introduction

The ability of stored product pests to remain hidden in the fabric of buildings is well known, with some species shown to demonstrate refuge-seeking behaviour in the laboratory (Coombs and Freeman, 1955; Liscombe and Watters, 1962; Cox *et al.*, 1989, 1990, 1993; Barson, *et al.*, 1992). Structural cracks and crevices may become filled with food residues, and provide nourishment and shelter for the pests. Residual treatments may fail to kill insects in these harbourages, allowing them to survive for longer periods and emerge later to re-infest foodstuffs (Pinniger, 1974; Jacobson and Pinniger, 1982). The use of repellents or flushing agents to treat refuges may increase the mobility of insects causing them to move out of their harbourages and make greater contact with residual treatments. An effective repellent may, therefore, enhance the efficacy of a residual treatment, and in the context of this project, increase fungal conidia uptake.

Alternatively, the contact between the insects and the conidia could be improved by attracting insects to areas where the conidia are present. Much work has been undertaken on developing attractants for stored product beetles. It should, therefore, be possible to attract insects to a bait station containing conidia for use in a lure and kill strategy. In this part of the project the potential

for moving the insects to the conidia either by the use of repellents to remove insects from cracks and crevices, or by attracting insects to a bait station containing conidia, were examined.

Removing insects from refuges

There appears to have been no successful use of repellents to protect stored grain from insect infestation on a commercial scale (Cox, 2004), although there has been increased interest in the use of plant-derived repellents (Ignatowicz and Wesolowska, 1994; Xie *et al.*, 1995; Weaver and Subramanyam, 2000; Papachristos and Stamopoulos, 2002; Tapondjou *et al.*, 2005). This has been fuelled by the need to investigate effective alternatives to conventional pesticides due to health, environmental and resistance concerns. However, it is sometimes erroneous to assume that naturally derived compounds are safer than conventional pesticides. The advantages and disadvantages related to the use of plant extracts in storage situations was summarised by Adler *et al.*, (2000) and are shown below:

Advantages	Disadvantages
Biodegradability	Low persistency
Broad-spectrum activity	Development of resistance possible
Good image among customers	Changing contents of active compounds
Low cost “do-it-yourself” production in countries of origin	Registration as plant protectants difficult
	Potential toxicity to man
	In food: no neutral taste

The effects of plant extracts on stored product pests is often quite complex, with different species and developmental stages reacting differently to a given extract (Adler *et al.*, 2000). Vast arrays of plants have been evaluated against stored product pests. Most information has been produced from developing countries seeking low cost, locally available plant products. However, many studies have lacked standardised techniques, such as those described by Loshciavo (1952), Laudani and Swank, (1954) and McDonald *et al.* (1970).

Examples of plants shown to have repellent properties against stored product insects include extracts from *Ocimum* spp. (basil), *Capsicum annum* (chilli), *Curcuma longa* (turmeric), *Eucalyptus saligna*, coriander and *Mentha* spp. (mint) (Su, 1986; Trematerra and Sciarretta, 2002; Chander *et al.*, 2000; Obeng-Ofori and Reichmuth, 1997; Tapondjou *et al.*, 2005; Aggarwal, *et al.*, 2001).

For naturally occurring repellents to be considered for use in U.K. grain stores, they should be effective against a range of pest species in typical storage conditions, have low mammalian toxicity, be easy to apply and readily available and have approval for such a use. With these points in mind, two compounds were selected for further consideration.

Diatomaceous earths

Although diatomaceous earths (DEs) may not strictly be considered plant-derived compounds, they comprise fossilised skeletons of diatoms, which are microscopic plants closely related to brown algae.

In stored product protection, DEs have proved effective as grain protectants (Desmarchelier and Dines, 1987; Subramanyam *et al.*, 1994) and structural treatments to storage facilities (Bridgeman, 1994, 2000; Wright, 1990; Desmarchelier *et al.*, 1992; McLaughlin, 1994). The products contain no chemical insecticide or knock-down agents, have low mammalian toxicity, do not leave harmful residues, are effective against chemical-resistant species, are persistent and are stable at high and low temperatures (Subramanyam *et al.*, 1994; McLaughlin, 1994). As well as direct toxic effects, DEs have also been shown to have repellent properties (White *et al.*, 1975). Grain treated with 0.01% DE was repellent against *S. oryzae*, *T. castaneum* and *C. ferrugineus* (Mohan and Fields, 2002). A DE-tolerant strain of *T. castaneum* was seen to avoid grain treated with DE at concentrations of 75 ppm, whereas, a susceptible strain did not avoid DE at 600 ppm (Rigaux *et al.*, 2001).

DEs have also been shown to synergise the effect of *Beauveria bassiana* on stored grain beetles (Akbar *et al.*, 2004; Lord, 2001). Therefore, beetles exposed to DEs in refuges may be more susceptible to the effects of the fungus, thereby requiring less exposure or lower fungal doses to produce an effect.

Although DEs have low mammalian toxicity, the main health concern is from inhalation of the dust. DE products used in pest control normally contain <1% crystalline silica, making them safe to the operator, providing a suitable dust mask is worn. In the UK, the Health and Safety Executive have set the long-term (8-hour) occupational exposure standard (OES) for DE at 1.2 mg/m³ (Anon, 2002).

Pyrethrins

The only plant-derived insecticides used on a large-scale on stored commodities are pyrethrins, obtained from the flowers of *Chrysanthemum* sp (Weaver and Subramanyam, 2000). The main use of natural pyrethrins in food processing areas, in combination with piperonyl butoxide, is similar to that used in household situations where an aerosol is used to knock-down insects. Pyrethrin sprays have the additional effect of stimulating insects to move rapidly and come out of hiding, and are therefore used as flushing agents against insects such as cockroaches. Pyrethrins have also been shown to be repellent against stored product insects (Khan, 1983; Haliday and McGovern, 1987; Egwunyenga *et al.*, 1998). Adults of *T. confusum* reared on media treated with sub-lethal

doses of synergised pyrethrins increased their resistance to the compounds but were still significantly repelled when applied to paper and food media (Cline *et al.*, 1984).

The advantages of pyrethrins include low mammalian toxicity and rapid breakdown and to date, stored product insects have not developed high levels of resistance. Although natural pyrethrin is still used commercially, it has largely been superseded by synthetic pyrethroids, which have improved insecticidal properties and photostability. Pyrethrins have approval for amateur use as an insect spray on edible crops and as a fly spray in animal houses.

The difficulty in using repellents to treat refuges in grain stores is the ability to treat all refuges successfully, rather than merely repelling insects from one treated refuge to an untreated one. An appropriate dose also needs to be determined so that insects are repelled and not killed, although having repellent compounds that are also toxic may be beneficial in enhancing the effect of the fungi. The aim of these experiments was to assess the repellency and mobility effects of the compounds in the laboratory by recording insect behaviour in response to filter paper halves treated with each compound, using specialised 'Ethovision' software and to determine the ability of the compounds to remove insects from refuges.

A. Repellency and mobility effects of the chosen compounds

Materials and Methods

Insects

Known aged adults (1-3 weeks) of a laboratory organophosphate resistant strain of *O. surinamensis* (Tram) were used. Prior to testing, individual insects were removed from the laboratory culture and held singly in glass tubes, without food, overnight.

Test compounds

Diatomaceous earth (DE): Silico-sec, a natural DE containing 90% SiO₂, was applied at the recommended application rate of 10g/m².

Pyrethrins: A ready to use formulation of Pybuthrin 33 containing 3g/l pyrethrins (including cinerins) was applied at the recommended rate of 5l/100m², equivalent to 150 mg a.i./m². This product is a ready to use space and surface spray and is suitable for use in food handling premises. It has a rapid knock-down and kill action and also flushes out insect from harbourages. Ethanol was used as a diluent.

N,N-diethyl-m-toluamide (DEET): A technical sample containing 97% active ingredient was applied at 250 mg/m², using ethanol as a diluent. DEET was included in the testing as this compound is a

known insect repellent and was used to monitor a positive repellent response by the insects and to act as a positive control for the test method.

Bioassay method

The repellency of each compound was assessed using a technique similar to that described by Laudani *et al.* (1955). Filter papers (7 cm in diameter) were cut in half with one half designated as the 'treated' half and the other the 'untreated' half. Ethanol (0.2 ml) was applied evenly to the 'untreated' half of each paper used to assess DEET and pyrethrin. DEET and pyrethrin solutions (0.2 ml) were then applied evenly to their respective 'treated' halves. The effect of ethanol was assessed by having control papers with one half treated with ethanol and the other untreated. The filter papers were transferred to a pin board and dried in a fume cupboard overnight.

The DE papers were prepared on the day of testing to prevent air movements re-distributing the dust over the paper. Whole papers were used with the 'untreated' half remaining untreated. The required amount of DE was sprinkled as evenly as possible over the 'treated' halves ensuring that no dust went on to the 'untreated' half, by covering the untreated half with another filter paper half during treatment.

For each treatment, four replicates were prepared for each compound for each day of testing. Individual filter paper halves were placed together on a glass sheet and held in place with a metal ring (5.5 cm in diameter) the inside surface of which was coated with Fluon. Each replicate was arranged so that each treated half was rotated through 90° compared to the adjacent replicate, to overcome any directional influences. One beetle was then placed in the centre of each replicate. The beetles were allowed to settle for 5 minutes in the arena before their movement was recorded for 60 minutes using a video camera positioned above the filter papers. New beetles were used for each test replicate. Experiments took place on 4 separate days with the order of treatments randomized on each day. All experiments were done in a controlled environment room at 20°C ± 2°C and 70% ± 5% r.h. in darkness.

The behaviour of the beetles was analysed using 'Ethovision' software and the mean total time, % duration, mean distance travelled and mean velocity on each half of the filter paper were determined. The time spent and distance travelled on each half were analysed using ANOVA (at the 5% probability level) to determine if the beetles spent significantly less time and were more mobile on the treated halves, which may suggest that they had been repelled by the compounds.

Results

The mean total time, % duration, mean distance travelled and mean velocity of beetles on each filter paper half is shown in Table 6. There was no significant difference ($P > 0.05$) in the time the

beetles spent on the untreated and ethanol halves, the ethanol and pyrethrin halves and the DE and untreated halves, suggesting that the pyrethrin and DE were not repellent to the beetles. The beetles did, however, spend significantly ($P<0.05$) less time on the DEET half compared to the ethanol half, indicating the beetles were repelled by the compound as may be expected. There was also no significant difference in the results obtained on different days indicating that there was no day effect.

Table 6. Mean total time, % duration, mean distance travelled and mean velocity of *O. surinamensis* on each half of filter paper treated with different compounds (n=16)

Treatment	Mean time (min) spent on each half with 95% confidence limits	Mean % duration on each half	Mean distance (cm) travelled with 95% confidence limits	Mean velocity (cm/min) on each half
Control				
Untreated half	27.8 (24, 31.6)	46.3	389.4 (278.9, 499.8)	14
Ethanol half	31.7 (27.7, 35.8)	52.8	422.1 (319.2, 524.9)	13.3
DEET				
Ethanol half	34.6 (31.6, 37.5)	57.7	390 (318.5, 461.5)	11.3
DEET half	25.2 (22.2, 28.2)	42	318.5 (254.4, 382.6)	12.6
Pyrethrin				
Ethanol half	29.9 (26.2, 33.6)	49.8	411.6 (276.9, 546.4)	13.8
Pyrethrin half	30.1 (26.4, 33.8)	50.2	379.9 (276.7, 483.2)	12.6
DE				
Untreated half	31.6 (26.4, 36.8)	52.7	190 (135.2, 244.8)	6
DE half	28.4 (23.2, 33.6)	47.3	116.6 (83.9, 149.4)	4.1

There was also no significant difference ($p>0.05$) in the distance travelled by and the velocity of beetles on the untreated and ethanol halves, the ethanol and pyrethrin halves and the ethanol and DEET halves, suggesting that these compounds did not affect mobility. The beetles did, however, travel significantly ($P<0.05$) shorter distances and were slower on the DE treated half, compared to the untreated half. Also, compared to the other treatments, the beetles travelled significantly less and were slower on both the DE treated and untreated halves, suggesting that exposure to the DE affected the mobility of the insects in some way which continued once away from the DE. There was no significant difference in the results obtained on different days with the controls, DEET and pyrethrin indicating no day effect. However, on day one, the beetles travelled significantly greater distances on the DE treated halves compared to the other three days.

Discussion

Under the conditions of these experiments, the results suggest that the pyrethrin and DE compounds were not repellent to *O. surinamensis* adults at the doses assessed. It may be that

because the insects had been starved prior to testing, their foraging behaviour in search of food may have overcome any repellent effect of the compounds. A longer exposure period may also have increased the repellency, as some pesticide resistant strains of *O. surinamensis* have demonstrated delayed avoidance behaviour beyond 2 hours, when exposed to organophosphate and pyrethroid treated papers (Watson and Barson, 1996).

In some of the DE treated replicates, there was a definite avoidance of the DE treated half as shown by the tracking profile; however, this was not consistent throughout the experiment. DEs have been shown to have repellent properties against *Sitophilus oryzae*, *Tribolium castaneum* and *Cryptolestes ferrugineus* when applied to wheat (Mohan and Fields, 2002; Rigaux *et al.*, 2001). Silica has also been shown to be repellent to *T. castaneum* when applied to filter papers (Willey, 1984). The DE did, however, cause the beetles to move slower and cover less distance compared to the other compounds, even when away from the treated surface. This suggests that the DE affected insect movement in some way and that dust particles picked up on the treated side continued to affect the insects when on the untreated side. During recording, it was observed that the beetles appeared to move tentatively through the DE. This may be a disadvantage in the context of this project as reduced mobility will reduce the amount of fungal conidia disseminated to other insects, but may enhance fungal pick up as insects will be in contact with the conidia for longer compared to a product that is repellent.

The beetles appeared to be little affected by the presence of the pyrethrins, moving freely between the two halves of filter paper. Adult *T. castaneum* have been shown to avoid surfaces treated with 0.2% pyrethrins, although avoidance was not demonstrated by *S. granarius* (Prickett and Ratcliffe, 1977). Adults of *Tribolium confusum* reared on media treated with sub-lethal doses of synergised pyrethrins, were significantly repelled by the compounds when applied to paper and food media (Cline *et al.*, 1984).

B. Ability to remove insects from refuges

Materials and methods

Arenas were formed by sealing a steel ring (20 cm diameter) to a Whatman no. 1 filter paper (27 cm diameter) using instant Polyfilla®. Refuges were composed of two, 7cm square glass plates (1.5mm thick) that were separated by a segment of nylon washer (~1.5mm thick) in each corner. This created a crevice between the glass plates that insects could enter. Filter papers (Whatman no.1), treated with the test compound, were glued to the internal surfaces of the glass plates using a proprietary adhesive (Pritt™) and trimmed to the correct size. DE (Silico-sec™) was applied to papers after they were fixed to the glass plates. All treatments were applied at the equivalent recommended rate. Four refuge treatments were examined: Control (blank), Silico-sec (dust) (10mg m⁻²), pybuthrin (insecticide) (150 mg m⁻²) and kerosene (solvent).

Adults were placed in the refuge with a small quantity (~0.75g) of the standard culturing media (wheatfeed, rolled oats & yeast (5:5:1 by weight)) within arenas. Observations were made every 15 min for the first hour, every 30 min for the next 2 hours and hourly for a further 2 hours. A final assessment was made after 24 hours. The number of insects in the arena was recorded at each observation point

Results

Analysis using Anova by GLM on transformed ($\sin^{-1}\sqrt{p}$) data indicated that there were differences between the treatments (T) (df=3, 396, F=38.54, P<0.001), time (t) (df=10, 396, F=214.99, P<0.001) and the interaction (T x t) (df=30, 396, F=4.86, P<0.001). These differences become most apparent after an hour. After this period, fewest adults were observed in arenas with control refuges, and the most for treatments in which the refuges contained Silico-sec. Pybuthrin and kerosene treated refuges resulted in a similar response, intermediate to the control and dust refuges (Table 7). The number of insects in the arena generally decreased over time for all treatments.

Table 7. The mean percentage of *O. surinamensis* observed in arenas containing refuges with different treatments at various time points after introduction (N=5).

Elapsed Time	Control			Silico-sec			Pybuthrin			Kerosene		
	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
00:15	89.4	98.0	76.0	79.0	92.0	72.0	87.4	94.0	84.0	87.0	92.0	78.0
00:30	85.6	96.0	80.0	86.4	90.0	82.0	84.6	90.0	76.0	87.8	96.0	80.0
00:45	82.2	94.0	72.0	84.0	92.0	78.0	84.2	88.0	78.0	83.6	94.0	74.0
01:00	80.2	94.0	74.0	81.0	88.0	74.0	81.0	86.0	70.0	86.2	94.0	78.0
01:30	59.8	80.0	28.0	74.4	82.0	60.0	59.8	68.0	38.0	59.8	76.0	46.0
02:00	42.4	56.0	16.0	61.2	78.0	44.0	49.4	68.0	34.0	48.0	62.0	34.0
02:30	39.8	56.0	18.0	61.6	70.0	56.0	46.2	62.0	32.0	47.4	64.0	36.0
03:00	36.2	50.0	14.0	60.6	66.0	52.0	49.0	66.0	34.0	45.4	62.0	32.0
04:00	33.6	46.0	18.0	53.8	68.0	40.0	45.2	58.0	34.0	43.8	58.0	26.0
05:00	35.2	54.0	20.0	54.0	64.0	46.0	43.8	62.0	28.0	50.4	68.0	42.0
24:00	21.2	32.0	14.0	54.4	64.0	36.0	36.6	52.0	20.0	36.4	46.0	24.0

Discussion

Some species of stored product beetle, including *O. surinamensis* have been shown to exhibit refuge-seeking behaviour (Coombs and Freeman, 1955; Liscombe and Watters, 1962; Cox *et al.*, 1989, 1990, 1993; Barson, *et al.*, 1992) and this may serve as a mechanism by which insects are able to avoid contact with pesticide treated surfaces. The presence of a repellent compound within a refuge could, therefore, optimise contact with treated surfaces. At the concentration tested the

diatomaceous earth, Silico-sec, reduced the number of insects that were present in the refuge. This was noticeable one hour after the insects were introduced to the arena. Pybuthrin and kerosene also reduced the number of insects present in the refuge, but not to the same extent as the diatomaceous earth. The repellency of diatomaceous earths to some pest insect species has been reported previously (Mohan and Fields, 2002; Alves *et al.*, 2008). Although the number of insects present in the crevice at the observation times was reduced in comparison to the control, insects were still present in the refuge. The length of time that an individual spent in the refuge was not examined, and it is possible that insects entering the refuge remained there for less time than insects in the control treatments. The work has shown that, on a small scale, it is possible to reduce the number of insects present in a refuge at a given time. However, the ability to achieve this at a larger scale remains to be determined and practical issues with regard to treatment of all potential refuges may preclude this as a practical measure to improve uptake by the insects.

C. The potential of bait stations

Materials and methods

Arenas consisted of an aluminium ring (20 cm diameter), coated with fluon, sealed to a 27 cm diameter Whatman no. 1 filter paper using quick drying instant Polyfilla®. The base of a 9 cm diameter glass Petri dish was used as the bait station. This was placed in the centre of the arena. Treatments used bait stations either with or without dry conidia powder of isolate IMI 389521. The dry conidia powder was spread evenly over the base of the Petri dish. In addition, to determine whether the presence of an attractant lure would improve contact with the conidia, a lure developed to attract several species of stored product beetle (Collins *et al.*, 2007; 2008) was placed in some of the 'bait stations', with and without the conidia. For each of the four treatments (no conidia, no lure; no conidia, with lure; conidia, no lure; conidia, with lure) there were ten replicates. Adult *O. surinamensis* (20) were put into each arena and left for 48 hours. During the first 24 hours, arenas were covered with foil with a damp filter paper attached to the side covering the arena. This was to provide increased humidity. After 48 hours, the insects were removed from the arenas and transferred to 90 mm diameter Petri dishes containing a 50 mm diameter filter paper and a small amount of culture food. Mortality was assessed 14 days after the experiment was set up. Tests were conducted at 20°C 70% r.h.

Results

Control mortality (treatments without the conidia) was very low and, as expected, there was no significant difference between control treatments with and without the lure ($P > 0.05$) (Figure 24). There was a significant difference between treatments with and without the conidia in the bait station (GLM, $F_{3,36}$, $P < 0.001$). Mortality was significantly higher in treatments with the conidia. Mortality in the treatment with the lure in the bait station with the conidia was significantly greater than for the conidia without the lure ($P < 0.05$) (Figure 24)

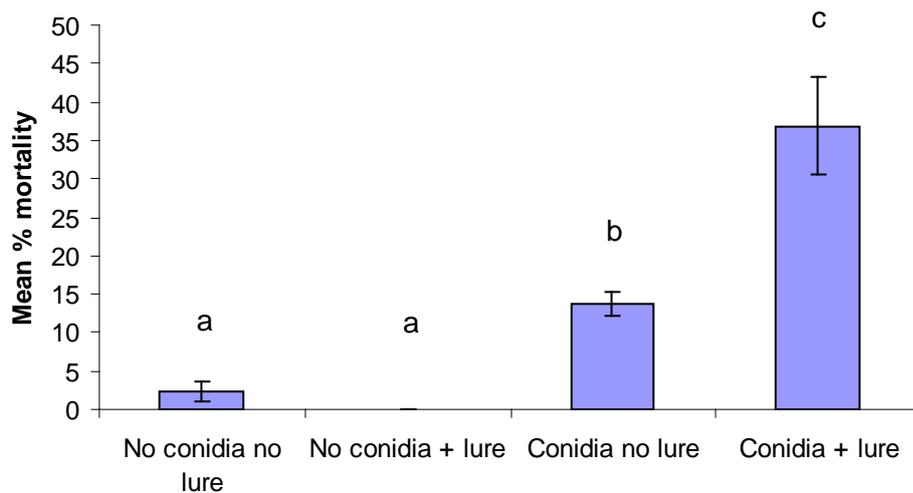


Figure 24. Mean (\pm SE) % mortality 14 days after exposure to different treatments in 'bait stations' (N=10).

Discussion

The use of bait stations to deliver a lethal concentration of the fungus to the pest insect would enable targeted delivery of the biopesticide formulation. This study has shown that insects will enter an area where the conidia are present and will pick up a lethal dose of the dry conidia powder. The presence of an attractant lure in the bait station resulted in an increase in the mortality of *O. surinamensis*. It was noted when insects were transferred from the arenas to the Petri dishes after 48 hours that more insects were present in the bait station with the lure in comparison to the bait station without the lure (Wakefield, personal observation). The lure may, therefore, have had an arrestant effect in addition to acting as an attractant. In a supplementary study, using refuges constructed as described in Section B, insects were provided with a choice of a refuge, with or without conidia present. The study showed that mortality in the arenas with a choice of a refuge with or without the conidia was not significantly different ($P > 0.05$) to the mortality of insects in arenas where both refuges contained conidia (results not shown). It would, therefore, appear that the insects did not avoid the refuge with the conidia when a choice was available. Bait stations containing an appropriate formulation of the fungal isolate offer potential of targeted delivery of the conidia to the insects.

Moving the conidia to the insects

Introduction

To complement the studies aimed at moving the insects to the conidia, opportunities to move the conidia to the insects were examined. The study investigated the prospects of using an inert powder, EntostatTM (Exosect Ltd, Winchester, UK), as a carrier for the fungal conidia. Insect cuticles carry a small electrostatic charge that is built up during walking and flight activity (Berry, 1973; McGonigle and Jackson, 2002; McGonigle *et al.*, 2002). Electrostatically charged powders,

such as Entostat, which contains micronised carnauba wax, can adhere to the charged insect cuticle and can be formulated with active ingredients (Howse and Underwood 2000) including insecticides, entomopathogens, pheromones or essential oils. Moths and fruit flies visiting dispensers are able to take up Entostat on their cuticles and transfer Entostat between individuals during physical interaction (Barton *et al.* 2006; Baxter *et al.* 2008; Armsworth *et al.* 2008). Entostat has also been used as a carrier and disseminator of entomopathogenic fungal conidia within beehives to control varroa mites (Meikle *et al.* 2007). Baxter (2008) demonstrated that the proportion of conidia uptake by *Plodia interpunctella* from treated tubes was better in an Entostat formulated product than plain conidia with no carrier. The aim of these studies was to evaluate the potential of Entostat to adhere to stored grain beetles when applied to refuges and determine whether the insects are repelled by Entostat, as other studies have shown that insects may be repelled by inert dusts (Glenn *et al.*, 1999).

In this study, *O. surinamensis* was used as a model species to examine uptake and behavioural responses to Entostat. Regression analysis was used to model Entostat uptake and retention by the beetles. Scanning electron micrograph (SEM) images were collected to investigate Entostat retention on different body parts. A behavioural experiment was conducted to establish at what level Entostat mixed with rolled oats appeared to repel *O. surinamensis* individuals. A three-choice experiment was also conducted in which cracks contained untreated oats, oats mixed with 5% (w/w) Entostat, or oats mixed with 5% (w/w) Entostat and a piece of filter paper containing a beetle attractant. The purpose of this experiment was to determine whether the beetle attractant would increase the attractiveness of the crack treated with Entostat. Finally, we examined the adherence of fungal conidia to a variety of dry powders, including Entostat, under a scanning electron microscope (SEM) to assess the suitability of Entostat as a carrier. Diatomaceous earth and kaolin clay were selected for comparison with Entostat as all powders comprise fine particulate matter used currently in other methods of insect control.

Materials and methods

Insects

Oryzaephilus surinamensis were obtained from the Food and Environment Research Agency, York (formerly the Central Science Laboratory) culture and reared on rolled oats (95% w/w) and brewers yeast (5% w/w). The culture was maintained at 28°C, 50-70% r.h. and ambient light conditions. Adults used in all experiments were of unknown sex, mating status and age.

Powder uptake

A fluorescent dye, Glo-Brite AW Powder (Himar, Bradford, UK) (10% w/w) was dissolved in ethanol and formulated into Entostat. Throughout this report, “dyed Entostat” refers to the formulated mixture of Entostat, “food mixture” refers to rolled oats mixed with dyed Entostat, while “clean

rolled oats” refers to oats without dyed Entostat. Using a scanning fluorometer (Perkin Elmer Luminescence Spectrometer LS50B), we found that Glo-Brite has an optimum excitation wavelength of 385 nm and emission wavelength of 450 nm, respectively. A calibration curve was constructed testing the emission from six concentrations of powder in ethanol: 0.1, 0.2, 0.4, 0.8, 1.5 and 1.9 µg of dyed Entostat per 1 ml 95% ethanol. In order to quantify Entostat uptake, *O. surinamensis* individuals were transferred to Eppendorf tubes containing 95% ethanol and vortex-mixed for 30 s before the solution was analysed in the fluorometer.

Uptake and retention of Entostat in rolled oats

The purpose of this no-choice experiment was to determine to what extent uptake and retention of dyed Entostat was associated with the amount to which the beetles were initially exposed. Rolled oats were mixed with dyed Entostat in the following percentages (w/w): 0 (clean oats), 2, 5, 10, 20, and 30%. For each concentration, 0.5 g food mixture (rolled oats and dyed Entostat) were placed in a 5 cm-diameter Petri dish and 10 *O. surinamensis* individuals were introduced for 24 h. After 24 h, the 10 beetles in each dish were transferred to a new 5 cm-diameter Petri dish with 0.5 g clean rolled oats. Beetles were kept in the clean rolled oats for 24, 48, and 72 h before being transferred as a group to a 1.5 ml Eppendorf tube with 1 ml of 95% ethanol for quantification of Entostat uptake. All combinations of dosage of Entostat in food mixtures and time in clean oats after treatments were repeated 15-25 times.

Behavioural response to Entostat

The purpose of this choice experiment was to determine whether beetles were repelled by the presence of dyed Entostat in oats. Artificial cracks were created by inserting a 5 cm long piece of 2 mm thick white PVC tubing into another piece with a 2 mm larger inner diameter so that the space between the two pieces constituted a crack (Figure 27b). Tape was used in the bottom to hold the two pieces together and prevent the food matrix from coming out of the crack. Consequently, beetles could only access cracks from the top. These trials were conducted inside 200 ml transparent plastic containers. The food mixtures (0.5 g) described above were added to cracks and 0.5 g of clean rolled oats were placed in the centre of the tubes. Consequently, beetles could choose between treated food inside the crack and untreated food in the centre. Between 10-15 beetles were released in the centre, and after 48 h, the numbers of beetles found in the centre, the crack, or outside the two pieces of PVC were recorded. This experiment was repeated 10 times for each concentration of dyed Entostat in food mixtures.

Three-choice experiment

The purpose of this three-choice experiment was to determine dyed Entostat uptake when beetles were offered choices between treated and untreated cracks. Three cylindrical cracks (as described above) were placed in plastic boxes (3 litre volume), but this time no food was added to the centre.

Of the three cracks, one contained clean rolled oats (“Oat”), the second contained 95% rolled oats and 5% dyed Entostat (“Treated 1”), and the last crack contained 95% rolled oats, 5% dyed Entostat, and 10-20 mg of a beetle attractant (Collins and Chambers, 2003), which was applied to 2 cm² filter paper and inserted into the crack (“Treated 2”). Forty-eight hours after being released, the number of beetles in each crack was recorded. Beetles outside the three cracks were grouped as “Outside”. Beetles in each of the four groups (Oat, Treated 1, Treated 2, and Outside) were transferred in groups to Eppendorf tubes for fluorometric analysis. The dyed Entostat estimate from the fluorometric analysis was subsequently divided by the number of beetles in each group. This experiment was repeated 10 times and 20-25 beetles were used in each trial.

Conidia and carrier powder admixtures

Diatomaceous earth and Kaolin clay were either mixed directly with dry attenuated *Beauveria bassiana* conidia provided by CABI or they were formulated with coatings of stearic acid or carnauba wax using proprietary manufacturing methods at Exosect. Entostat was mixed directly with conidia. All seven powder types were mixed with fungal conidia at a ratio of 100:1 (w/w) and examined using SEM.

Ten *O. surinamensis* were exposed to rolled oats containing 5% (w/w) of the Entostat and conidia mixture for 48 h in Petri dishes, and were then removed to clean rolled oats for 24 h. Individuals were then examined using SEM.

Scanning Electron Microscopy

For analysis of beetles treated with plain Entostat, beetles were kept in 5 cm-diameter Petri dishes containing rolled oats and 5% Entostat for 24 h and subsequently transferred to Petri dishes with clean rolled oats for 24-72 h. Before imaging, beetles were glued onto aluminium stubs (Agar Scientific Ltd., Stansted, UK) and viewed directly without further preparation using a FEI Quanta 200 scanning electron microscope (FEI Company, Cambridge, UK) in variable pressure mode (10 Kv and 60 Pascal). Beetles were examined at various magnifications. Beetles treated with the Entostat and conidia mix were also mounted and viewed in the same way. For examination of the conidia/powder admixtures, a thin coating of each powder was applied to aluminium stubs using a paintbrush and viewed directly as for beetles at various magnifications.

Statistical analysis

Uptake and retention 24-72 h after exposure to dyed Entostat was examined in a regression analysis using the following curve fit (Sigmaplot 8.0, SPSS Inc., Chicago, Illinois, USA):

$$\text{Equation 1: } Ent = a \times \exp^{-0.5 \times \left(\left[\frac{P-P_0}{b} \right]^2 + \left[\frac{T-T_0}{c} \right]^2 \right)}$$

in which amount of powder on individual beetles (*Ent*), is described by two independent variables, percentage of dyed Entostat mixed into the food (*P*) and time since exposure (*T*), and P_0 , T_0 , a , b , and c are fitted coefficients.

Within the PROC MIXED procedure in PC-SAS 9.0 (SAS Institute Cary, NC, USA), one-way ANOVA with contrasts was used to analyse the level of repellency to dyed Entostat. The same procedure was used to compare amounts of dyed Entostat on beetles in the three-choice experiment. Also, in the three-choice experiment, a Chi-square test was used to determine whether the distribution of beetles among the four groups (Oats, Treated 1, Treated 2, and Outside) was significantly different from that expected from an even distribution.

Results

Uptake and retention of Entostat in rolled oats

The first part of the fluorometric analysis addressed quantification of two background levels of fluorescence: auto-fluorescence of *O. surinamensis* and fluorescence from the food source itself. Fluorometric analysis of beetles kept in empty Petri dishes suggested that auto-fluorescence of *O. surinamensis* for the examined wavelength (450 nm) was equivalent to an estimate of Entostat uptake $<0.010 \mu\text{g}$ (Table 8). In addition, in the positive control (beetles kept in clean rolled oats) the level of fluorescence of oats itself was negligible, as readings per beetle were $<0.010 \mu\text{g}$. Consequently, it was established that fluorescence readings equivalent to an estimate of Entostat uptake $>0.010 \mu\text{g}$ could be attributed to uptake and retention of Entostat. Depending upon initial Entostat concentration in the food source, 0.03-0.26 μg powder was extracted from individual beetles 72 h after being transferred from treated to untreated food sources. Using equation 1, the combination of percentage of dyed Entostat and time since exposure provided a significant curve-fit to uptake by beetles (Adjusted $R^2 = 0.712$, $F_{273} = 169.46$, $P < 0.001$) (Figure 25). This suggested that initial uptake of dyed Entostat reached an asymptotic level with about 25% dyed Entostat mixed into the food source.

Table 8. Average (s.e.) amount (μg) of Entostat on individual 24, 48, and 72 h after exposure

Time	Blank	Percentage rolled oats in food mixture (rest is dyed Entostat)					
		100	98	95	90	80	70
24	0.003(0.015)	0.003(0.013)	0.041(0.068)	0.192(0.386)	0.208(0.322)	0.862(1.269)	0.977(0.909)
48	0.007(0.023)	0.005(0.015)	0.012(0.025)	0.050(0.068)	0.134(0.441)	0.584(0.697)	0.596(0.47)
72	0.000(0.002)	0.000(0.002)	0.026(0.056)	0.207(0.453)	0.111(0.288)	0.276(0.532)	0.263(0.432)

Considerable amounts of dyed Entostat powder were taken up and retained for up to 72 h by *O. surinamensis* individuals, and powder retention is likely associated with a fairly high density of hairs on most body parts (Figure 26a and b). From scanning electron microscope analysis, we found

Entostat powder on all body parts, but generally insertions of hairs (Figure 26c), and intersegmental areas (Figure 26d) were particularly suitable sites for powder deposition. Entostat particles were also seen on the elytra (Figure 26e), but the largest concentrations of Entostat were found in areas just behind the front legs, where Entostat was creating a thick layer (Figure 26f).

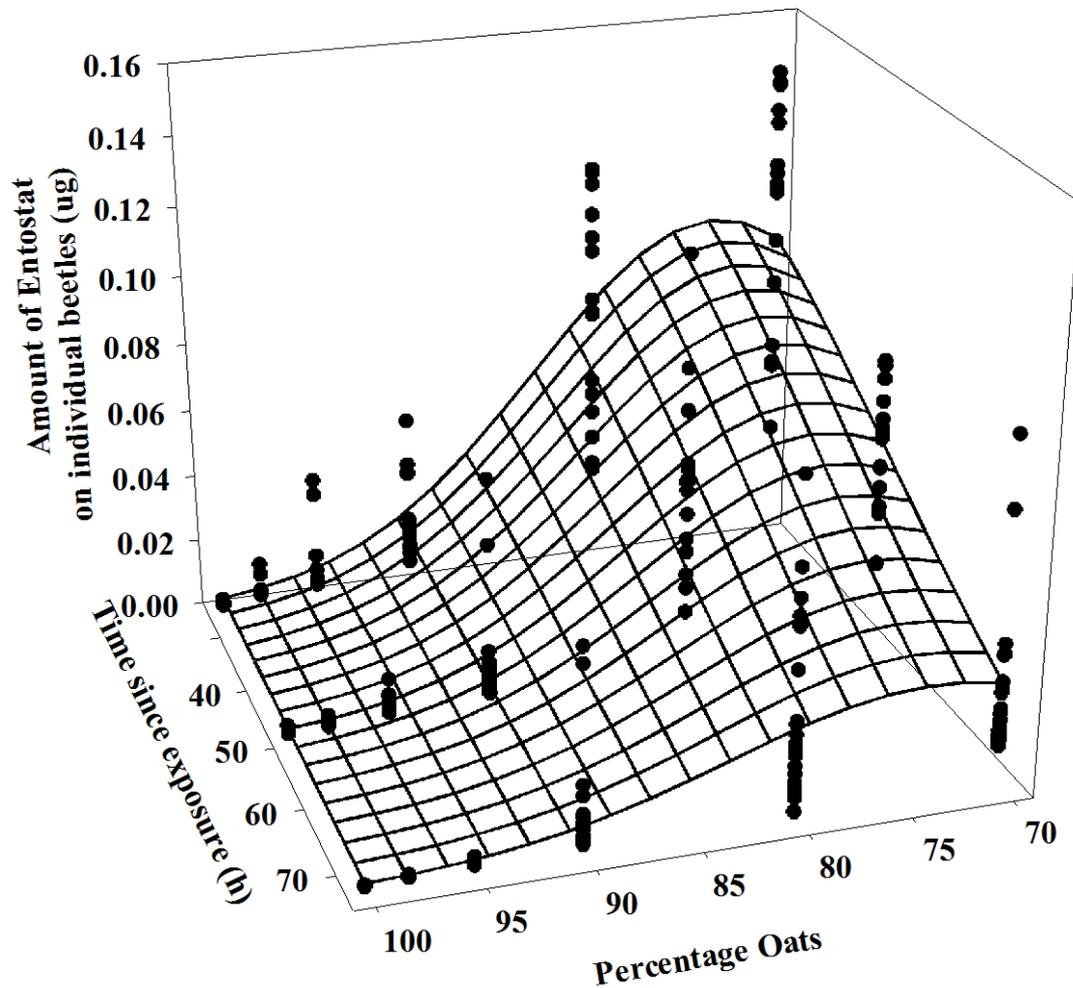


Figure 25. Curve fit of amount of Entostat on *Oryzaephilus surinamensis* individuals (Ent) 24-72 h after exposure to 0-30% dyed Entostat [based on Equation 1: $Ent = 0.11 \cdot \exp(-0.5 \cdot (((P - 73.60)/9.47)^2 + ((T - 9.25)/39.88)^2))$].

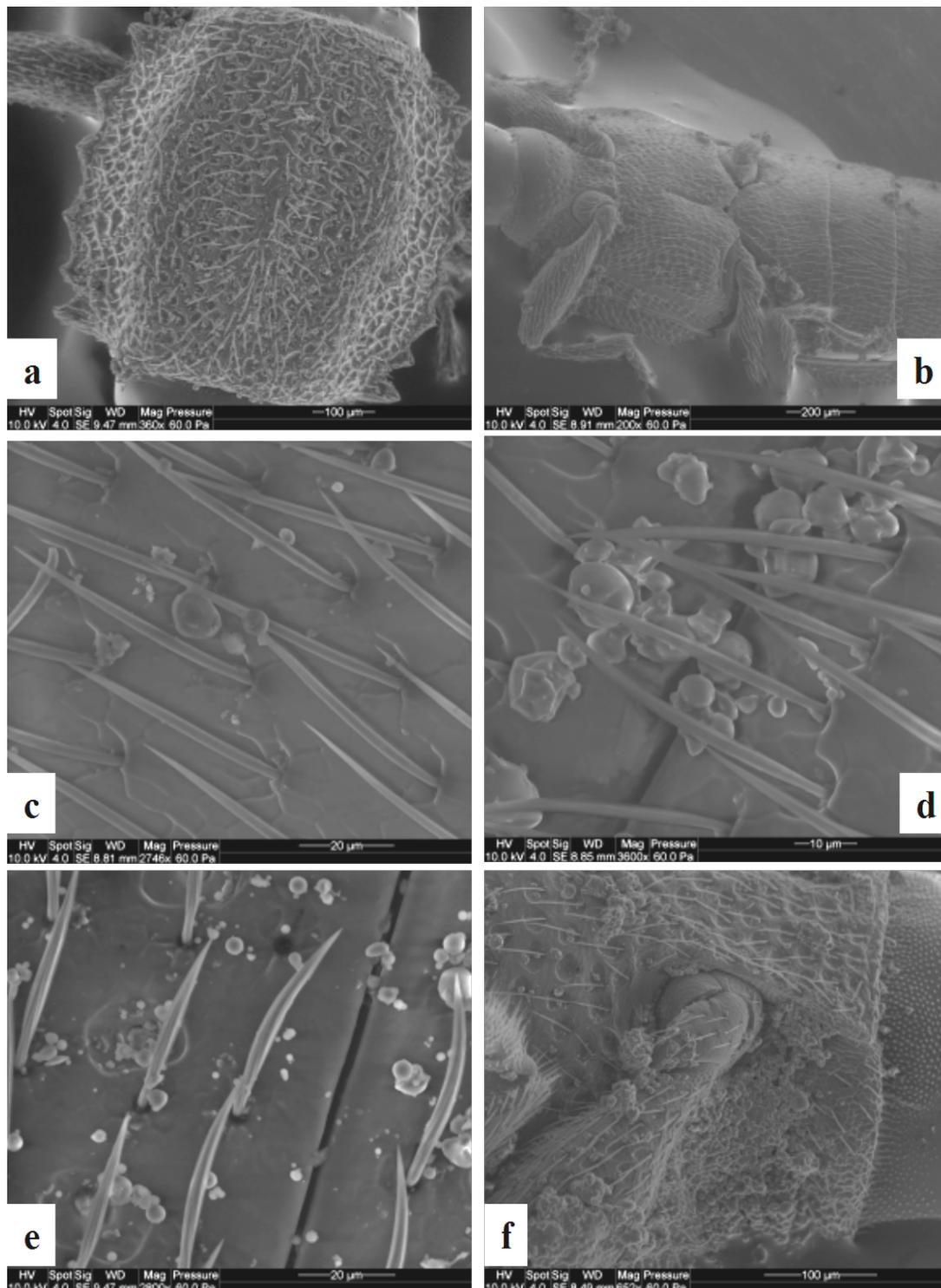


Figure 26. Scanning electron microscope images of *Oryzaephilus surinamensis* individuals taken after being kept for 24 h in rolled oats with 5% dyed Entostat and subsequently kept in untreated food for 24 h (a) or 72 h (b-d). Images show pronotum (a), ventral side of body (b), and spherical Entostat particles on ventral side of abdomen (c), and intersegmental areas on ventral side of abdomen (d), elytra (e) and behind front legs (f).

Behavioural response to Entostat

Across trials with different concentrations of dyed Entostat in a crack and clean rolled oats in the centre (Figure 27a), about 20% of beetles were found outside the two food sources (centre and crack) (Figure 27b). In trials with clean rolled oats both in the crack and in the centre, about 50% of

beetles were found in the crack and 30% in the centre. Increasing the amount of dyed Entostat in the treated crack caused gradual decrease in proportion of beetles inside the crack, with only about 15% found in cracks with 30% dyed Entostat. In the one-way ANOVA of proportions of beetles found in treated cracks, there was a significant effect of Entostat dosage ($F_5 = 11.49$, $P < 0.001$). There was no significant difference in proportions of beetles found in treated cracks with 0-5% dyed Entostat (Pairwise contrasts $P > 0.05$), but higher dosages of dyed Entostat caused a significant decrease in proportions of beetles in treated cracks. Thus, it was decided to conduct further experiments on the basis of 5% dyed Entostat in cracks.

Three-choice experiment

With three different cracks and 10-25 beetles used in each trial, an average of 4.85 beetles would have been expected in each of the four groups (Oat, Treated 1, Treated 2, and Outside), if these were evenly distributed (Figure 28a). However, beetles were not found in all four groups in all 10 trials: for instance, in Treated 2, beetles were only found in cracks in four of the 10 trials, and there were three trials in which no beetles were found in cracks with Oats and Treated 1. Significantly more beetles (about 80%) were found outside cracks ($DF = 10$, $\text{Chi}^2 = 69.28$, $P < 0.001$). Only 4-10% of beetles were found in any of the three cracks, which in all three cases was significantly lower than expected ($P < 0.05$). In a second Chi-square analysis, beetles not found in cracks were excluded, which meant that an average of 1.47 ± 0.43 beetles would be expected in each of the three cracks; this value was not significantly different from observed in any of the three groups ($P > 0.05$). Average amounts of dyed Entostat were significantly higher on beetles found in Treated 2 cracks compared to beetles from any of the other groups (Oat, Treated 1, and Outside) ($F_3 = 27.40$, $P < 0.001$) (Figure 28b). We found no significant difference in the amount of dyed Entostat on beetles from Oat, Treated 1, and Outside ($P > 0.05$). The amount of dyed Entostat on beetles from Oat, Treated 1, and Outside was between 0.05-0.10 μg , which is within the expected powder retention after 48 h from treatments with 5% dyed Entostat (Table 8). Thus, our data suggests that although beetles may not have spent much time in dyed Entostat-treated cracks (Treated 1 and 2), they picked up considerable amounts of dyed Entostat and retained it even after moving to other parts of test arenas.

SEM Examination of conidia and carrier powder admixtures

Kaolin clay powder and diatomaceous earth (DE) comprised variable size ($< 5 \mu\text{m}$) and shape irregular particles and few conidia were seen adhering to the exterior of the kaolin or DE particles; many conidia remained loose within both the mixtures (Figure 29a and d). When kaolin and DE were formulated with a coating of stearic acid, particle size was increased and the mixtures became clumpy but adherence of the conidia appeared improved (Figure 29b and e). A coating of carnauba wax caused the kaolin powder to form large clumps with some particle sizes $>100\mu\text{m}$; however, conidia were seen to adhere all over the exterior of these particles. When DE was coated

in carnauba, particle size was increased, but it was not as clumpy as the carnauba-coated kaolin; the conidia adhered well to the coated particles (Figure 29c and f). Adherence of the conidia was better when kaolin and DE were coated with carnauba wax than stearic acid, perhaps because micronised carnauba wax is known to exhibit electrostatic properties. All coated formulations would require additional processing to reduce particle size and clumping. When Entostat alone was mixed with the conidia then the conidia were observed to adhere all around the outer surfaces of the spherical wax particles (Figure 30a and b); this was also the easiest formulation to manufacture because it was directly admixed without first applying a coating to the wax.

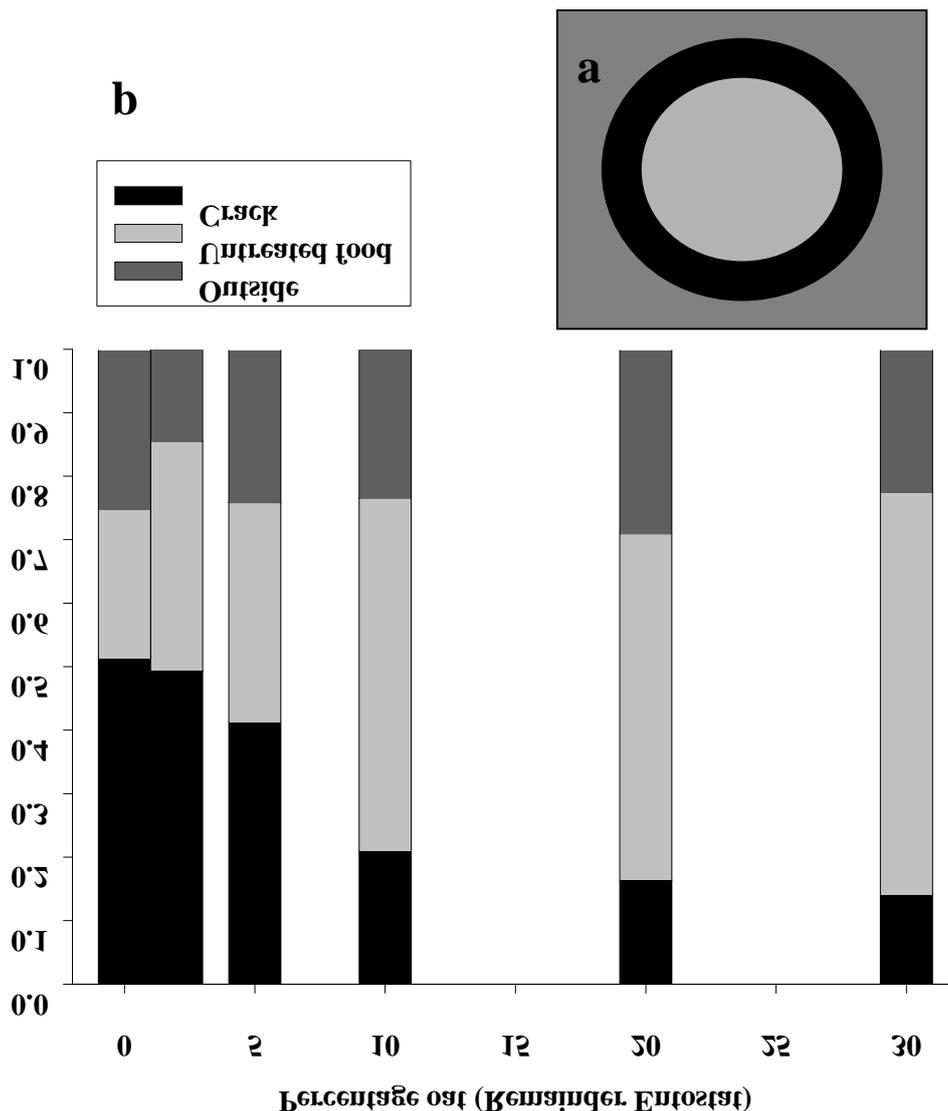


Figure 27. Diagram showing how two pvc tubes were used to create a circular “crack or crevice” (a). Proportion of *Oryzaephilus surinamensis* individuals in centre, crack and outside after 48 h, when crack contained various levels of Entostat (b).

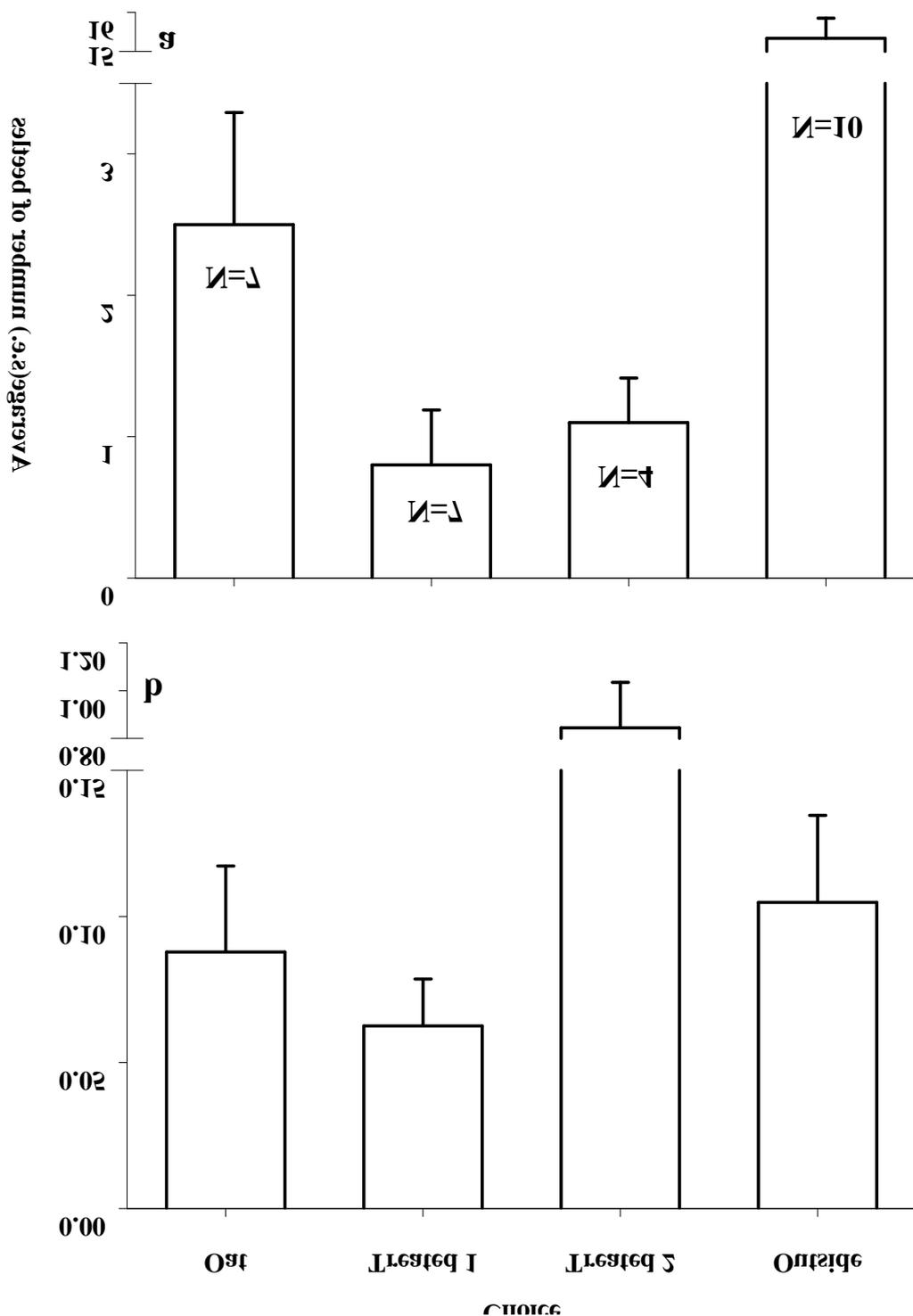


Figure 28. Three circular “cracks or crevices” (see Fig. 3a) were used in choice test: “Oat” denotes untreated rolled oats, “Treated 1” denotes rolled oats with 5% (w/w) dyed Entostat, and “Treated 2” denotes rolled oats with 5% (w/w) dyed Entostat and a piece of filter paper with 10-15 mg of a beetle attractant. Number of *Oryzaephilus surinamensis* individuals in each of the three cracks or outside (anywhere else) after 48 h (a), average amount of dyed Entostat on beetles (b).

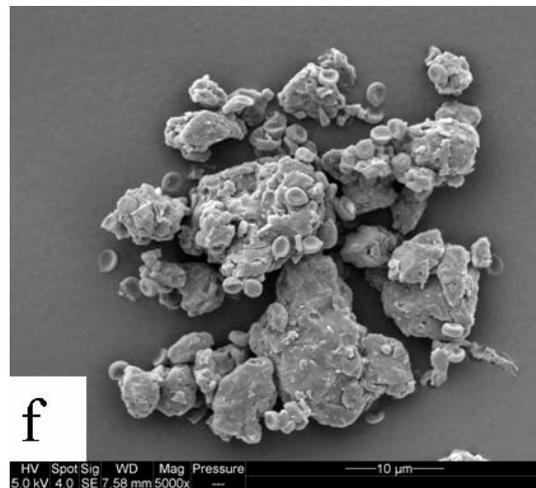
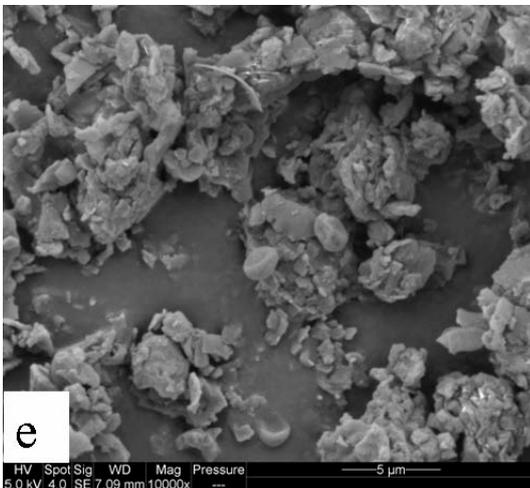
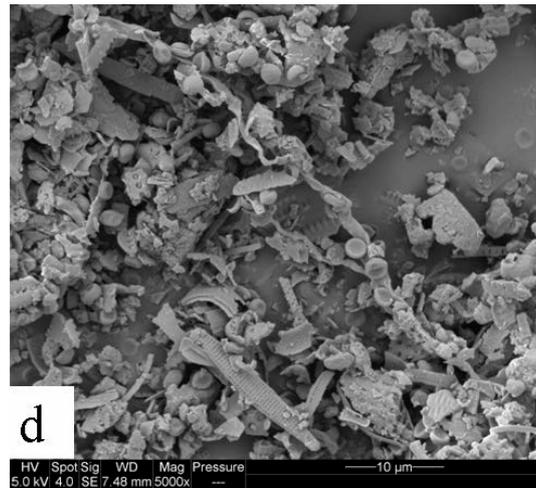
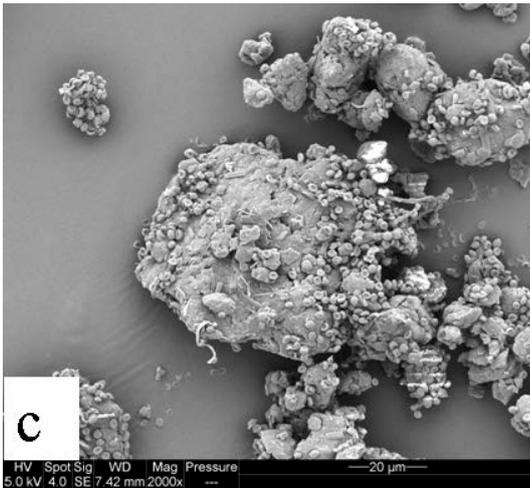
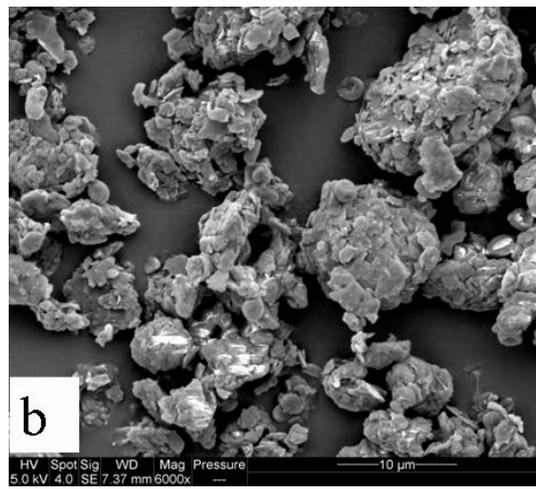
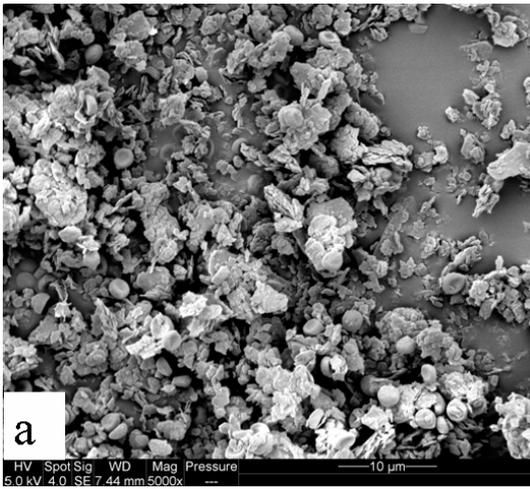


Figure 29. Scanning electron microscope images of *Beauveria bassiana* conidia admixed with (a) kaolin, (b) kaolin coated with stearic acid, (c) kaolin coated with carnauba wax, (d) diatomaceous earth, (e) diatomaceous earth coated with stearic acid and (f) diatomaceous earth coated with carnauba wax. Conidia are identifiable as 2-3 µm concave discs.

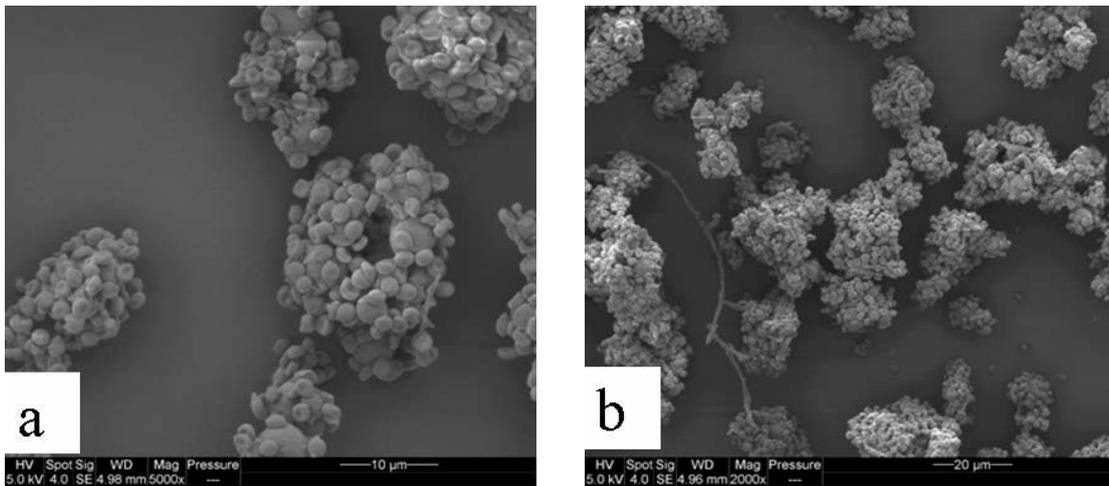


Figure 30. Scanning electron microscope images of *Beauveria bassiana* conidia admixed with (a and b) Entostat. Conidia are identifiable as 2-3 µm concave discs.

Conidia and Entostat were easily identified adhering to the cuticles of *O. surinamensis*, particularly in cuticle indentations and structures such as sensilla pits (Figure 31a and b). We also observed that the conidia were no longer adhering to the exterior of the Entostat particles but appeared now to be adhering directly to the insect cuticle.

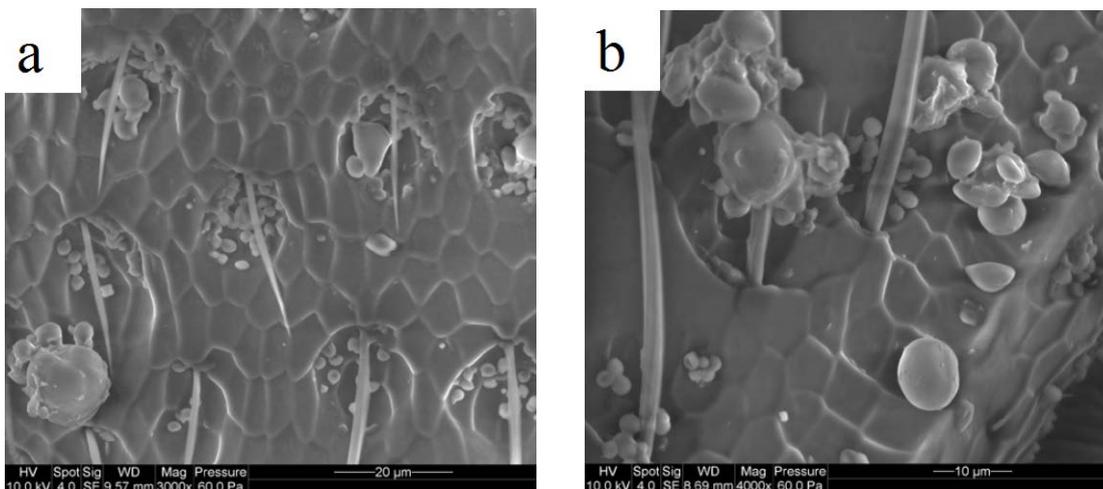


Figure 31. Scanning electron microscope images of *Oryzaephilus surinamensis* cuticle after exposure to rolled oats treated with an Entostat and *Beauveria bassiana* conidia mixture (a and b). Entostat and conidia can be clearly seen adhering to the insect cuticle, particularly around the sensilla pits. Entostat are identifiable as 2-10 µm spherical particles and conidia as 2-3 µm concave discs.

Discussion

In this study, we showed that Entostat uptake and retention by *O. surinamensis* can be modelled using two explanatory variables, the amount of dyed Entostat added to the food mixture and the time since exposure. The same analysis showed that dyed Entostat uptake became asymptotic at about 25% (w/w) Entostat in food mixtures. After keeping beetles in untreated food for 72 h, beetles initially kept in food containing 5% dyed Entostat were shown to be contaminated with

>100 ng of powder. *Oryzaephilus surinamensis* appeared to be repelled by dyed Entostat when cracks contained >5% dyed Entostat so this study suggested that 5% should be a target dosage for this carrier to be used in crack and crevice treatments. In a three-choice experiment, we showed that although the majority of beetles were found outside treated cracks, the average amount of dyed Entostat per beetle was >50 ng, independently of where beetles were found. It appeared that the CSL attractant did not significantly increase the attractiveness of the crack in which it was applied, but the average powder uptake of beetles from cracks treated with the attractant was significantly higher than from the other cracks. Thus, it is suggested that although the tested attractant may not have worked as a medium-range attractant (i.e. attracted beetles from 3-15 cm away) it worked as an arrestant for beetles that entered treated cracks and thereby caused higher uptake of dyed Entostat. Trematerra *et al.* (1999) tested *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and highlighted the possible importance of arrestants being emitted from broken wheat kernels. Arrestants for *O. surinamensis* have been identified (Nakajima *et al.*, 1997). It is also possible that the attractant somehow masked the repellency of dyed Entostat or in other ways increased the likelihood of beetles interacting more with the food mixture, which ultimately caused significant increase in powder uptake. Regarding the three-choice experiment, it is important to emphasize that the arena was fairly small (400 cm²) and that results might vary compared to similar studies conducted in larger arenas.

Conidia had a greater affinity for Entostat than two other fine powders: diatomaceous earth and kaolin clay, which are used in other methods of pest control. Coating DE and kaolin clay with a wax coating of either stearic acid or carnauba wax did appear to increase the level of adhesion between conidia and the powder; however, a lot of clumping was observed and particle sizes ended up much larger than the unprocessed DE, kaolin or Entostat. Conidia adhered to the exterior surfaces of the spherical Entostat particles by unknown adhesive mechanisms, possibly through static attraction as the Entostat is an electrostatically chargeable bipolar powder (Howse & Underwood 2000; Baxter 2008). The affinity of the conidia and Entostat provide evidence that Entostat could act as a suitable carrier for the conidia, and Entostat is already known to readily adhere to insect cuticles (Armsworth *et al.* 2006; 2008; Barton *et al.* 2006; Nansen *et al.* 2007), including *O. surinamensis* as shown in the present study by means of fluorescent assay and SEM imagery. Interestingly, the SEM images of *O. surinamensis* treated with an Entostat and conidia admixture showed that the conidia detached from the Entostat particles and attached to the insect cuticle. Thus, despite the excellent level of adherence of conidia to Entostat, the conidia clearly had a greater affinity for the insect cuticle than the Entostat particles. The attachment mechanism for conidia to cuticle could be more powerful than for conidia to Entostat. Boucias *et al.* (1988) demonstrated that hydrophobicity of the insect cuticle and conidial wall appeared to mediate adhesion rather than electrostatic forces. The ability of a carrier powder to co-locate with the

conidia and then to detach from conidia when in contact with the insect are desirable attributes of a conidia delivery system.

The current studies showed that, in multi-choice arenas, beetles acquired and retained similar amounts of dyed Entostat as in a no-choice experiment. Regarding powder uptake by insects, air movement and movement of insect pests within a food matrix may statically friction-charge the Entostat particles (tribo-charging) and thereby enhance adherence to insects - thus the insects themselves are contributing to their contamination with the powder. More research is needed to determine to what extent Entostat adheres to food debris surfaces and/or structural surfaces when it is applied into a cracks or crevices. Further experiments with Entostat and fungal conidia are described in other sections of this report.

3.3.2. Step 2.2 Subsequent spread of infection

Introduction

Dispersal of a fungal infection within an insect population is an important consideration to establish maximum effect. After initial infection, conidia may be passed to other uninfected insects by two mechanisms: secondary transmission and secondary cycling. Secondary transmission results from the physical contact with infected live individuals, whereas secondary cycling occurs after contact with dead mycosed insects. Both processes have the potential to enhance the infection rate by affecting individuals that were not in contact with the initial infection, thereby reducing the need for repeat applications and blanket coverage. Insects dying within refuges may have the potential to spread conidia to healthy insects that have remained protected in harbourages from the treatments. Previous studies demonstrated the potential of secondary cycling as a means of disseminating fungal conidia (Cox *et al.*, 2004).

Some storage beetle species have been shown to exhibit refuge-seeking behaviour (see for example Cox *et al.*, 1990). This behaviour can limit contact with residual pesticides but may be advantageous for dissemination, both initial and subsequent, of the biopesticide. However, it is possible that insect behaviour may be altered subsequent to infection or at sub-lethal concentrations.

These experiments aimed to investigate further the ability of secondary cycling and secondary transmission to enhance infection rates in conditions likely to be encountered within a grain store in the U.K and to establish whether refuge-seeking behaviour of infected insects is altered.

Potential for secondary cycling and secondary transmission

Materials and methods

Secondary cycling studies

Batches of *O. surinamensis* (Tram) were removed from the laboratory culture and treated with conidia (IMI 389521), suspended in sterile water containing 0.05% Tween 80, at a concentration of 1×10^8 conidia/ml using the dip test technique described in Step 1.2. Treated insects were placed on a dry filter paper in a plastic Petri dish (90 mm in diameter) with a damp filter paper placed in the Petri dish lid. The Petri dishes were held in the test conditions of 20°C and 70% r.h. for 24 hours before the filter paper was removed and food added. After 7 days any dead insects were surface sterilised with a 5% solution of sodium hypochlorite and were put onto a damp filter paper on the base of a Petri dish. The dishes were again returned to 20°C and 70% r.h. to await fungal sporulation. After 4-7 days insects were observed to have started sporulating. Batches of live uninfected insects were then removed from the laboratory culture and mixed with dead sporulating insects in a Petri dish, in the proportions below:

- 20 live uninfected insects (controls)
- 15 uninfected insects : 5 dead sporulating insects
- 10 uninfected insects : 10 dead sporulating insects
- 5 uninfected insects : 15 dead sporulating insects

Five replicates were prepared for each test condition. Conditions likely to be encountered in a practical situation in the UK were assessed; with temperatures of 15°C and 20°C and relative humidities of 70, 80, 90 and 100% at each test temperature. Food was added to each dish after 24 hours and mortality was assessed after 7, 10 and 14 days exposure. The dead insects were then surface sterilised with a 5% solution of sodium hypochlorite and were put onto a damp filter paper on the base of a Petri dish. The dishes were returned to 20°C and 70% r.h. and the insect assessed for sporulation after 7 days.

Secondary transmission studies

Batches of *O. surinamensis* Tram were removed from the laboratory culture and marked with a dot of poster paint applied to the dorsal surface of the thorax. The next day further batches of insects were removed from the laboratory culture and treated with conidia, suspended in a 0.05% Tween solution, at a concentration of 1×10^8 conidia/ml using the dip test method. Immediately after treatment, the marked untreated insects and the unmarked treated insects were added to a Petri dish in the following proportions:

- 20 marked untreated insects (controls)
- 15 marked untreated insects : 5 unmarked treated insects
- 10 marked untreated insects : 10 unmarked treated insects
- 5 marked untreated insects : 15 unmarked treated insects

- 20 unmarked treated insects

Five replicates were prepared for each test condition. Insects were exposed at temperatures of 15°C and 20°C and relative humidities of 70, 80, 90 and 100% at each test temperature. Food was added to each dish after 24 hours and mortality was assessed after 7 and 14 days exposure. Dead insects were surface sterilised with a 5% solution of sodium hypochlorite and were put onto a damp filter paper on the base of a Petri dish. The dishes were returned to 20°C and 70% r.h. and the insect assessed for sporulation after 7 days.

Results and discussion

Secondary cycling studies

After 7 days exposure at 20°C, 0-10% of the initially uninfected individuals had died at all four relative humidities tested (Table 9). Mortality increased slightly after 10 days exposure with 0.4-14%, 0-19% and 3-20% at 70%, 80% and 90% r.h. respectively, and 8-90% mortality at 100% r.h. After 14 days exposure, 5-25%, 0-23% and 7-25% of uninfected insects had died at 70%, 80% and 90% r.h. respectively. At 100% r.h. after 14 days exposure, 88% of uninfected insects had died with the lowest proportion of sporulating insects (25%) with complete mortality with the highest proportions (50% and 75%) (Table 9).

Table 9. Mean % mortality of uninfected individuals (adjusted for control mortalities) following exposure to different proportions of sporulating dead insects at 20°C.

Experimental conditions	Proportions of live : dead insects	7 days exposure	10 days exposure	14 days exposure
20°C / 70% r.h.	15 live : 5 dead	0	0.4	5
	10 live : 10 dead	0	3.7	10
	5 live : 15 dead	0	13.9	25
20°C / 80% r.h.	15 live : 5 dead	0	0	0
	10 live : 10 dead	2.5	3.7	7.7
	5 live : 15 dead	5	18.9	23.1
20°C / 90% r.h.	15 live : 5 dead	0	3.3	8.3
	10 live : 10 dead	0	2.5	7.5
	5 live : 15 dead	10	20	25
20°C / 100% r.h.	15 live : 5 dead	0	8.3	87.8
	10 live : 10 dead	0	90	100
	5 live : 15 dead	0	90	100
15°C / 70% r.h.	15 live : 5 dead	0	16	23.8
	10 live : 10 dead	12	18	28.6
	5 live : 15 dead	4	8	34.7
15°C / 80% r.h.	15 live : 5 dead	2.7	9.3	14.5

	10 live : 10 dead	16	46	51
	5 live : 15 dead	12	40	42.8
15°C / 90% r.h.	15 live : 5 dead	5.3	17.3	35.8
	10 live : 10 dead	2	12	23.2
	5 live : 15 dead	0	24	39.4
15°C /100% r.h.	15 live : 5 dead	1.3	16	50.7
	10 live : 10 dead	0	22	50
	5 live : 15 dead	8	24	60

Significantly higher mortalities occurred at 100% r.h. compared to the other relative humidities, with the highest proportions of sporulating insects producing the highest mortalities in the uninfected insects. After 14 days, the insect food had become very mouldy at 100% r.h. and it was difficult to recover all the test insects. It was assumed that those not recovered had died as live insects were easily found.

These results differ from previous experiments which found that in the presence of 20% of infected individuals, 17% and 91% of healthy insects died within 10 days after 48 hours and 10 days exposure respectively, at 20°C and 70% r.h. (Cox *et al.*, 2004). The only difference with the present experiments was that a damp filter paper was placed in the Petri dish lid for the first 24 hours of exposure, which may have aided sporulation and conidia uptake. However, it would be expected that at 100% r.h. equivalent mortalities would have been achieved, but only 8% mortality was recorded at 20°C and 100% r.h. after 10 days exposure to 25% of infected individuals, although this did increase to 88% after 14 days.

After 7 days exposure at 15°C, low mortalities (0-16%) of the initially uninfected individuals were found at the four relative humidities tested (Table 9). As at 20°C, mortality increased after 10 days exposure with 8-18%, 9-46%, 12-24% and 16-24% mortalities at 70%, 80%, 90% and 100% r.h. respectively. After 14 days exposure, mortalities ranged from 24-35%, 15-51%, 23-39% and 50-60% at the respective humidities (Table 9). Apart from at 100% r.h., mortalities were higher at 15°C than at 20°C after 14 days exposure.

In the secondary cycling experiments at both temperatures, the majority of dead initially uninfected insects showed signs of fungal infection, indicating that secondary cycling had taken place.

Secondary transmission studies

Mortalities in the treatments at 20°C ranged from 16-40%, 17-44%, 12-39% and 18-60% after 7 days exposure at 70%, 80%, 90% and 100% r.h., respectively (Table 10). After 14 days exposure, mortalities had increased to 18-50%, 20-52%, 18-59% and 56-100% at the respective humidities. When the numbers of dead marked untreated insects were counted and compared to the control

mortalities, there was only a small increase in mortality (0-10%) at 70%, 80% and 90% r.h. At 100% r.h., there was a 14-32% increase in the mortality of uninfected insects with the highest mortality recorded with the lowest proportion of treated insects. The results suggest that, apart from at 100% r.h., secondary transmission had little effect on the mortality of uninfected insects.

Table 10. Mean % mortality of untreated individuals (adjusted for control mortalities) following exposure to different proportions of treated live insects.

Experimental conditions	Proportions of Untreated : treated insects	7 days exposure	14 days exposure
20°C / 70% r.h.	15 untreated : 5 treated	0	0
	10 untreated : 10 treated	1.1	3.4
	5 untreated : 15 treated	12.1	10.1
20°C / 80% r.h.	15 untreated : 5 treated	6	3.5
	10 untreated : 10 treated	3.2	6.4
	5 untreated : 15 treated	0	2.1
20°C / 90% r.h.	15 untreated : 5 treated	0.4	0
	10 untreated : 10 treated	0	0
	5 untreated : 15 treated	0	0
20°C / 100% r.h.	15 untreated : 5 treated	0	31.5
	10 untreated : 10 treated	0	28.3
	5 untreated : 15 treated	0	14
15°C / 70% r.h.	15 untreated : 5 treated	0	6.4
	10 untreated : 10 treated	0	12.8
	5 untreated : 15 treated	6.4	6.4
15°C / 80% r.h.	15 untreated : 5 treated	0	0
	10 untreated : 10 treated	0	5.4
	5 untreated : 15 treated	10.6	5.4
15°C / 90% r.h.	15 untreated : 5 treated	0.7	5.1
	10 untreated : 10 treated	4.1	7.2
	5 untreated : 15 treated	0	1
15°C / 100% r.h.	15 untreated : 5 treated	1	5.1
	10 untreated : 10 treated	3.1	9.3
	5 untreated : 15 treated	0	9.3

Mortalities in the treatments at 15°C ranged from 9-21%, 4-16%, 9-11% and 6-14% after 7 days exposure at 70%, 80%, 90% and 100% r.h., respectively (Table 10). After 14 days exposure, mortalities increased to 23-45%, 21-56%, 18-46% and 19-60% in the respective humidities. The majority of the mortalities recorded were with the unmarked insects that had been treated with the fungus. When the numbers of dead marked untreated insects were counted and compared to the

control mortalities, there was again only a small increase in mortality (0-13%) suggesting that secondary transmission had little effect on mortality.

In all the secondary transmission experiments at both temperatures, all but one dead insect showed signs of sporulation indicating that they had been infected with the fungus, whereas those dead in the controls were uninfected. This indicates that although secondary transmission did not greatly increase the mortality of the untreated insects compared to the controls, it did take place.

Treated insects in the secondary transmission experiments were placed immediately onto a dry filter paper and into the experimental conditions. There was no extra period of high humidity. The mortality of insects where all were treated was higher in these experiments compared to previous experiments at 80% r.h. Previous experiments recorded 9% and 0% mortality at 20°C and 15°C, respectively where insects were sprayed directly with a different isolate and had no period of humidification (Cox *et al.*, 2004). In the present experiments, 52% and 56% mortality was recorded at the respective temperatures and 80% r.h., suggesting that the isolate formulation used in these experiments was more effective.

These experiments have demonstrated that secondary cycling has the potential to disseminate a fungal infection to uninfected insects with the greatest effects in conditions of 100% r.h. However, at 15°C, secondary cycling had a greater effect at the lower relative humidities than at 20°C. These conditions are likely to be encountered in a grain store in the U.K. and demonstrate the potential of an infection being passed throughout a population. Secondary transmission was shown to have little effect suggesting that contact with the larger number of conidia from sporulating insects was more effective than those treated with the initial infection. Higher doses may, therefore, increase the effects of secondary transmission.

Conclusions

- Secondary cycling was more effective at disseminating the fungal infection than secondary transmission.
- 20°C and 100% r.h. were the most effective conditions for secondary cycling to take place. 100% mortality of uninfected insects was recorded with 50% and 75% of sporulating insects present.
- At 15°C, 100% r.h. was the most effective condition for secondary cycling to take place. A maximum mortality of 60% of uninfected insects was recorded with the highest proportion of sporulating insects.
- In secondary cycling experiments, there were higher mortalities at 15°C and 70, 80 and 90% r.h. (14-51%) compared to equivalent humidities at 20°C (0-25%) after 14 days.

- Secondary transmission had little effect on increasing mortality (0-13%) of uninfected individuals at 15°C and all relative humidities.
- Secondary transmission had little effect on increasing mortality (0-10%) of uninfected individuals at 20°C at 70%, 80% and 90% r.h.
- In secondary transmission experiments at 20°C and 100% r.h., a maximum mortality of 31% was recorded with the lowest proportion of treated insects.

Refuge-seeking behaviour of infected insects

Materials and methods

Batches of *O. surinamensis* (Tram) (20 per replicate) were removed from the laboratory culture and treated with conidia (IMI 389521), suspended in sterile water containing 0.05% Tween 80, at a concentration of either 1×10^8 conidia/ml or 1×10^6 conidia/ml using the dip test technique described in Step 1.2. The concentrations were chosen to represent known lethal and sub-lethal concentrations. Control insects were treated with 0.05% Tween 80 or received no treatment. Insects were placed on a dry filter paper in a plastic Petri dish (90 mm in diameter) with a damp filter paper placed in the Petri dish lid. The Petri dishes were held at the test conditions of 20°C and 70% r.h. for 24 hours. After this period insects were transferred to arenas formed from a 20 cm diameter steel ring sealed to a 27 cm diameter Whatman No. 1 filter paper using Polyfilla®. Each arena contained a refuge composed of two 7cm square glass plates separated by placing one quarter of a 2 mm thick nylon washer in each corner. The inner surfaces of upper and lower glass plates were lined with filter paper (Whatman no.1) held in position using proprietary solvent free glue (Pritt Stick™). Each refuge contained a small quantity of rolled oats as a food source. The number of insects in the arena was observed on an hourly basis for the six hours starting from 24 h and 96 h after the insects were introduced. After the conclusion of the 96-hour observations, live insects present in the arena and the refuge were removed and maintained in 9 cm diameter Petri dishes with a small quantity of food for a further 9 days. Insect mortality was then assessed. Insects were maintained at 20°C, 70% r.h. throughout the experiment. Five replicate arenas were tested for each treatment and the experiment was repeated on four separate occasions. The number of insects present in the arena was compared by ANOVA of the arcsine-transformed data followed by Tukey's method for multiple comparisons where appropriate. Where large variations in live insects were evident a weighted ANOVA ($2(\sqrt{n})$) was used.

Results

Observation of the number of insects present in the arena at six one-hourly periods 48 h post-treatment (Figure 32) showed that significant behavioural differences were detected between treatments (T) (df=3,532, F=10.01, P<0.001) and observation periods (O) (df=6,532, F=9.49, P<0.001), but no (T x O) interaction (df=18,532, F=1.07, P=0.383) was found. Multiple

comparisons revealed significant differences between lethally infected (1×10^8 conidia/ml) beetles and all other treatments ($P \leq 0.003$).

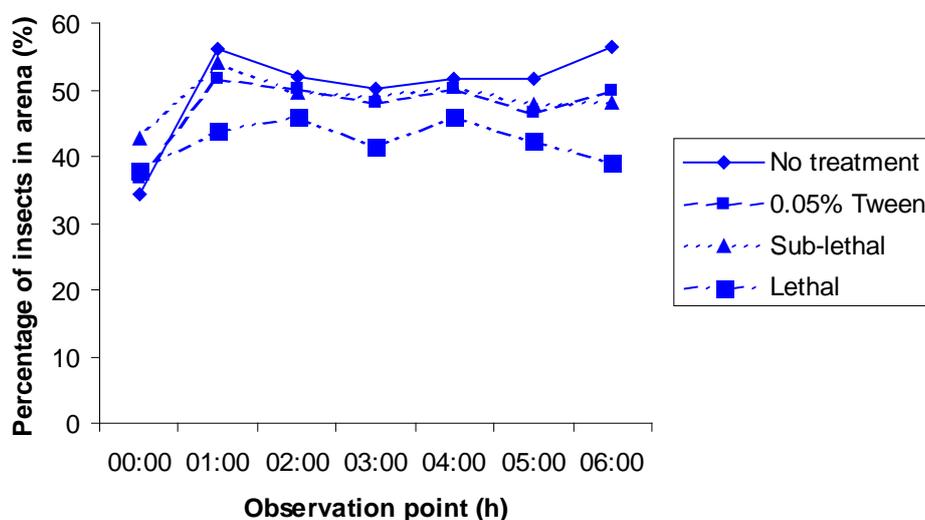


Figure 32. The proportion of insects present in an arena at six hourly observation points 48 h after treatment with a sub-lethal (1×10^6 conidia/ml) or lethal (1×10^8 conidia/ml) concentration of *B. bassiana* IMI 389521.

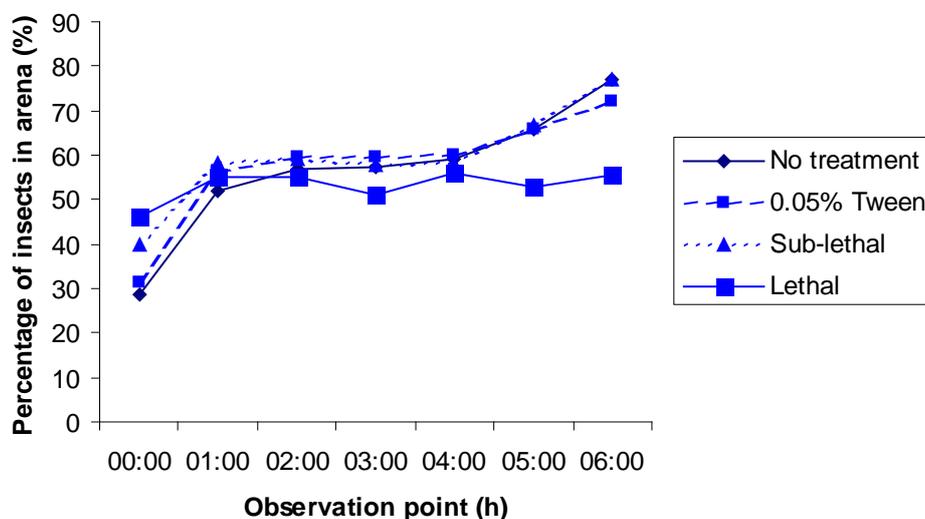


Figure 33. The proportion of insects present in an arena at six hourly observation points 5 days after treatment with a sub-lethal (1×10^6 conidia/ml) or lethal (1×10^8 conidia/ml) concentration of *B. bassiana* IMI 389521.

Observations 5 days post-treatment (Figure 33) using weighted analysis indicated that there were no behavioural differences between treatments (T) ($df=3,518$, $F=0.75$, $P=0.524$), but differences were detected between observation periods (O) ($df=6,518$, $F=16.05$, $P<0.001$); no significant interaction (T x O) was found ($df=18,518$, $F=0.08$, $P=0.991$). Fewer beetles were observed outside the refuge at the first observation ($P<0.001$) than at other periods; the final observations (6h)

differed from all others except the penultimate ($P=0.77$); no differences between the intermediate periods were detected ($P\geq 0.554$).

There was a significant increase in mortality of *O. surinamensis* treated with 1×10^8 conidia/ml compared with the other treatments (Table 11). The mortality did not increase greatly between 5 and 14 days for any treatment. A higher number of dead insects were found in the refuge in comparison with the number found in the arena. Overall, 90% of beetles survived the sub-lethal (1×10^6 conidia/ml) treatment compared to 12% for the lethal (1×10^8 conidia/ml) concentration.

Discussion

Oryzaephilus surinamensis exhibits refuge-seeking behaviour, which can reduce the uptake of a pesticide from a treated surface. However, this behaviour could be used to advantage in the development of a crack and crevice treatment.

Table 11. Mean (\pm SE) % mortality of insects removed from the arena or the refuge 5 days and 14 days post-treatment with IMI 389521.

Treatment	% Mortality at 5 days		% Mortality at 14 days	
	Arena	Refuge	Arena	Refuge
Control	0.25 \pm 0.25a	0.25 \pm 0.25a	0.25 \pm 0.25a	0.5 \pm 0.4a
1×10^6 conidia/ml	2.2 \pm 0.7a	5.1 \pm 1.1a	3.5 \pm 1.0a	5.9 \pm 1.2a
1×10^8 conidia/ml	29.0 \pm 3.3b	52.6 \pm 4.2b	30.1 \pm 3.4b	54.3 \pm 4.6b

In each column means followed by the same letter are not significantly different ($P>0.05$).

In this preliminary study to examine possible effects on insect behaviour the effect of a lethal and a sub-lethal dose of *B. bassiana* on the refuge-seeking behaviour of *O. surinamensis* was examined. Significantly fewer insects treated with the lethal concentration of *B. bassiana* were present in the arena at the observation points 48 h post-treatment in comparison with the other treatments. This indicates that a greater number of insects were present in the refuge. Laboratory bioassays have shown that insects infected with *B. bassiana* show only very low levels of mortality 72 h post-treatment (Wakefield, personal observation). Therefore, it is unlikely that the insects treated with the lethal concentration would have been dead at this time. The result indicates that insects infected with a lethal concentration of *B. bassiana* were less likely to leave the refuge than insects receiving the other treatments. After 5 days, many of the insects that had been treated with the lethal concentration were dead. Some of these were present in the arena and were therefore included in the observations, and the presence of these dead (immobile) insects would explain why no significant difference between treatments was observed five days post-treatment.

The number of dead insects found in the refuge was greater than that found in the arena. This could be of benefit in terms of secondary cycling if conditions within the refuge are suitable. It is likely that uninfected insects would enter the refuge containing the dead, sporulating insects, increasing the likelihood of infection.

Conclusion

- Insects infected with a lethal concentration of *B. bassiana* are more likely to remain in a refuge during the early stages of the infection process.
- Insects infected with a sub-lethal concentration of *B. bassiana* did not exhibit any changes in refuge-seeking behaviour.
- A greater number of insects died within the refuge, which may improve the potential for secondary cycling.

3.3.3. Step 2.3 Verifying the suitability of the chosen isolate

Introduction

In order for a biopesticide to be produced and used at a commercial scale, it is necessary to mass-produce the fungal isolate to obtain sufficient quantities. The mass production method must result in a product that is of a high quality and that is consistent between different batches. Sylvan-Somycel has led the development of the mass production method used in this project. During the process, different aspects of production were examined including the substrate, incubation conditions, extraction process etc. The specific details of the process are commercially sensitive, but an outline of the method is provided below.

Materials and methods

Rice was used as the substrate for the mass production method. The rice was cooked in an excess of boiled water until the moisture content desired, as indicated by the relative humidity, was obtained. A mineral powder was added to the rice after the cooking process. The substrate was sterilized in an autoclave prior to inoculation. After inoculation, the rice was placed in sterile culture bags. The cultures were incubated at 25°C until conidia production was obtained. After the growing period, the cultures were dried until the relative humidity of the rice was less than 15%. The fungal conidia were extracted and after extraction, the relative humidity of the conidia was adjusted to approximately 5%. Quality control checks were in place throughout the process. For the final product, the limits for an acceptable product were that the conidia count should be greater than 10×10^{10} conidia per gram of dry powder; germination should be greater than 80% and the product should be free of contaminants

Different batches, some of which were produced using slightly different methods during the development process, were tested against *O. surinamensis* Tram to determine the pathogenicity

(Table 13). The batches were tested using the dip test method, at a concentration of approximately 2×10^8 conidia/ml, and insects were maintained at 20°C, 70% r.h., without a period of increased humidity.

Results

The optimum mass production method was established following trials examining different aspects of the process. This has led to the production of dry conidia powder with very good quality control parameters (Table 12). The different batches of conidia were also found to result in a reasonably consistent level of mortality of *O. surinamensis* (Table 13).

Table 12. An example of the results from the quality control procedures for some of the batches of *B. bassiana* IMI 389521

Sample ID	Relative humidity	Conidia count / g of dry substrate (x 10e10)	Germination (%)
014	6	9.7	93
016	5	12	97
022	5.5	4.2	82.6
Trial 1	8	14	95
Trial 2	7.9	7.6	90

Table 13. Mean (\pm SE) % mortality 14 days after treatment with different batches of *B. bassiana* IMI 386243 or IMI 389521.

Sample ID	Mean (\pm SE) % mortality
521-003	13.0 \pm 1.2
521-021	34.1 \pm 6.5
521-022	30.6 \pm 6.0
521-023	34.7 \pm 6.2
243-017E	26.8 \pm 1.4
243-017R	30.3 \pm 3.1
243-018	44.0 \pm 6.9

Discussion

A mass production process that yields a consistent, high quality product has been established. Initial studies used both isolates IMI 386243 and IMI 389521. However, the viability and yields of batches of IMI 386243 were found to be more variable than for isolate IMI 389521. This finding, in conjunction with the data from Steps 1.1 and 1.3, demonstrating greater viability of IMI 389521 under conditions of low water activity and when formulated in different formulation types, led to the adoption of IMI 389521 as the preferred isolate. The establishment of the mass production method in the latter stages therefore focussed on isolate IMI 389521. The conidia produced caused low, but statistically significant, levels of mortality of *O. surinamensis* when tested using the dip test

method. This provided a base on which to establish the effectiveness of different formulation types on overall efficacy.

The development of the mass production method is an important step towards any future commercial use of a product. It is important that the fungus can be produced at an industrial scale and that the product meets established quality control parameters if it is to be used commercially. The scaling up of production from the laboratory was, therefore, an important step if the potential of a biopesticide as a structural grain store treatment is to be realised.

3.4. Objective 3. Consistency and safety

An essential part of the project was to show how the safety and quality of the putative biopesticide could be established such that there will be no future technical barriers to its subsequent registration. This work was undertaken in three steps. **Step 3.1:** The information that will ultimately be needed for subsequent registration of the biopesticide by industry was established. **Step 3.2:** The consistency of the material was confirmed by producing different batches of the chosen isolate and analysing them for compliance with specification standards. **Step 3.3:** Regulatory approval of a biopesticide will require assessment of possible risk due to non-target effects. These include those to occupationally exposed workers and to the grain being protected. The foundations were laid for the risk assessment process to be completed by industry after the end of this project.

3.4.1. Step 3.1 Requirements for registration of a biopesticide based on *Beauveria bassiana*

Introduction

Within the course of the project, information on the data that would be required as part of a registration package for a biopesticide has been acquired. In addition, a representative from the Chemicals Regulation Directorate (CRD; formerly the Pesticide Safety Directorate (PSD)) was appointed to the Project Management Group to monitor project progress and advice. Discussions with representatives from CRD, particularly with regard to efficacy testing were also held in the latter stages of the project. The following provides an overview of the data requirements for a registration package as they currently stand.

Legislation

Commercialisation of the proposed use in the European Union (EU) falls under the scope of the Plant Protection Product Directive, 91/414/EC.

An active substance is a Plant Protection Product (PPP) when it used to protect plants or plant products in their raw state or when they have undergone a simple process such as sawing, milling,

dehusking, etc. However, as soon as the plant or plant product has undergone an industrial process such as baking, cooking, curing, etc, the active substance, used to control a target organism for instance in store rooms, falls under the biocide directive.

Directive 91/414/EC was revised specifically to address the regulatory data requirements for a microorganism with the publication of EU Directive 2001/36/EC. This legislation falls into two parts. Part 1 describes the data required for the active ingredient. The European Commission have reviewed the definition of a microorganism in the context of Directive 2001/36/EC and ruled that this must be identified to strain level. Part 2 describes the data that must be supplied on the formulated product containing the microorganism.

EU registration

Two *Beauveria bassiana* strains have been reviewed at EU level and have been Annex I listed on Directive 91/414/EC (Table 14). This procedure enables registration of formulated products containing these strains at EU Member State (country) level.

The placing of these two microorganisms onto Annex I has closed the opportunity to establish a country registration for a product containing a different *B. bassiana* isolate. Any new isolates of *B. bassiana* (such as IMI 389521) will be considered as a new microorganism and will require evaluation of both the microorganism and the formulated product through the EU registration procedure. New microorganisms not registered previously in the EU can be notified and submitted at any time.

Table 14. *Beauveria bassiana* isolates with Annex 1 listing

Beauveria bassiana Strain identity	Use	EU Directive	EU review report
ATCC 74040	Control of whiteflies, thrips and aphids in tomatoes	2008/113/EC	SANCO/1546/08 rev3
GHA	Control of sucking insects in ornamentals, tomatoes, and cucumbers grown in greenhouses	2008/113/EC	SANCO/1547/08 rev3

Regulatory evaluation within the EU is a centralized procedure and the evaluation of the new microorganism, and its associated representative formulation, is conducted by an individual Member State authority (called the Rapporteur Member State, RMS). The two strains of *B. bassiana* that have achieved Annex I listing have been reviewed by the authorities in Germany. As this authority is now recognized as the 'expert' it must be considered most likely that they would be assigned as RMS for the IMI 389521 evaluation.

The evaluation of a new microorganism and its associated formulation represents an extensive data set and a detailed evaluation. Typical review times to achieve Annex I listing would be approximately 3 years. The cost of the evaluation would be in the range of €100,000 to €150,000. The cost to assemble a compliant dossier from an experienced regulatory consultant together with their time to manage any questions during the evaluation would be €20,000 to €50,000.

Data requirements

Specific and separate requirements have been established for the microorganism (annex II) and the formulated product (annex III). These are shown in EU directive 2001/36/EC.

Microorganism (annex II)

Studies should as much as possible be conducted according to international guidelines (such as those of US EPA Microbial Pesticide Test Guidelines, OPPTS Series 885, Feb. 1996). Where required, test guidelines for chemicals should be adapted, to make them suitable for microorganisms. Testing should include viable and, where appropriate, non-viable microorganisms and a blank control. All laboratory studies should in principle be conducted under GLP and field or greenhouse studies under GEP. However, the experience is that a study lacking GLP or GEP is not always rejected; this depends upon the evaluating authority and the type of study.

A valid scientific argument, why a particular study should not be required, can be used to replace any study. All available literature should be provided. Where possible, open literature can be used to meet any data requirement.

The most important and informative information is obtained from the characterisation and identification of the microorganism. The sections 1-3 (identity, biological properties and further information) form the basis for an assessment of human health and environmental effects. Toxicological data are required unless on the basis of existing information it can be shown that the use of the micro-organism under the proposed conditions does not have any harmful effects on human health, animal health or on ground water or any unacceptable influence on the environment.

When testing is done, the specification of the material should be provided, including any impurities, and the material should have the same specification as used for the manufacture of the product. The material used for studies should have the same specification as used for the large scale manufacturing, unless it can be shown that laboratory or pilot plant produced material is essentially the same as from the manufacturing plant.

When a study uses different doses, the relationship between dose and adverse effects must be reported.

In the evaluation of both the *B. bassiana* strains for Annex 1 listing, it was established that the level of the metabolite Beauvericin must not exceed 5mg/kg.

A listing of the key headings for annex II data requirements is provided in Appendix I of this report.

Formulated Product (annex III)

Studies should, as much as possible, be conducted according to international guidelines (such as those of US EPA Microbial Pesticide Test Guidelines, OPPTS Series 885, Feb. 1996). Where required, test guidelines for chemicals should be adapted, to make them suitable for microorganisms. When a study uses different doses, the relationship between dose and adverse effects must be reported. When testing is done, the specification of the material should be provided including any impurities and the material should have the same specification as used for the manufacture of the product.

In cases where a new preparation/formulation is to be dealt with, extrapolation from Annex II could be acceptable, provided that all the possible effects of formulants and other components, especially on pathogenicity and infectiveness, are also evaluated. This would also apply to the toxicological section.

A listing of the key headings for annex III data requirements is provided in Appendix II of this report.

OECD guidance

A harmonized set of data requirements for microbial pesticides has been developed by the OECD. This has been published in their document 'Guidance for registration requirements for Microbial Pesticides, OECD Series on Pesticides, Number 18, 2003'.

As a rule, regulatory authorities require sufficient information to characterize a micro-organism and assess its potential risk to people and the environment, whilst demonstrating acceptable efficacy. Typically, microorganisms have narrow host ranges and occur in nature in the environment in which they are to be applied. The risk assessment relies heavily on the biological and ecological profile of the microorganism with a set of short-term toxicity/pathogenicity studies. Such factors reduce the likelihood of this risk and consequently allow a high degree of confidence in the safety of the microorganism based on a reduced data set.

This position was revisited by an international panel of experts in 2008. They reviewed the safety evaluation systems paying particular attention to the characterization and identification of the microorganism, the efficacy of the product and the production of metabolites. The findings from the meeting have been published in the OECD document 'Working document on the evaluation of microbials for pest control, OECD Series on Pesticides 43, 2008'.

The first step in the risk assessment of a microorganism is to uniquely identify the organism. This issue has been addressed by the EU Commission and they have concluded that inclusion of microorganisms into Annex I should be at strain level. This position has been published in document SANCO/10754/2005 rev 5, Guideline developed within the Standing Committee on the Food Chain and Animal Health on the taxonomic level of microorganisms to be included in annex I to Directive 91/414/EEC. In practice, the core of the dossier will comprise strain specific information with additional relevant species-specific information. Notifiers are encouraged to make use of publicly available, previously assessed information.

This guidance advocates that the same Rapporteur Member State is used for the same species and that a pre-submission consultation with the Rapporteur is recommended.

The OECD panel in 2008 considered that only metabolites produced during the manufacturing process should be identified and quantified. This position has been confirmed in the Annex I listing for Bb strain ATCC74040 and GHA with a limit of 5mg/kg for the metabolite beauvericin.

Although not a requirement in some countries, data on efficacy is required in the EU. As a general principle, efficacy trials must be carried out under the conditions of use and the rates proposed on the label. However, for microbials the mode of action should be taken into consideration for efficacy trial design and assessment parameters should be selected accordingly. This issue was discussed in detail at the 2008 OECD meeting and it was recommended that efficacy trials follow the guidance published by EPPO on the 'Principles of acceptable efficacy'. The level of performance for a biological product such as a microorganism has been shown to be lower than a conventional PPP. It will, therefore, be important to establish the type of label claim proposed for the product formulated with IMI 389521. Products may be categorized as achieving 'control', 'suppression' or 'reduction' of a particular pest.

A standardized format has been developed for the submission of microorganisms and their products and is described in document 'OECD Guidance for industry data submissions for microbial pest control products and their microbial; pest control agents, OECD Series on Pesticides 23, 2006'. This guidance is broadly similar to that used for PPP and is the layout expected in all EU Member States.

3.4.2. Step 3.2: Consistency and safety of the mass-produced conidia

Consistency

Introduction

Wright *et al* (2001) stated that 'quality control is one of the most critical concerns in any industrial scale production system'. In order to maximise storage potential of an isolate, it has been reported that drying conidia before storage increases the amount of time which a product can be stored, and the lower the moisture content of conidia on storage, the longer the storage period (down to <5%) (Hong *et al.* 2001). Viability is also an important aspect to consider; when applying a biopesticide with a higher viability level, less conidia are required in the formulation as mortality is dose related. The LUBILOSА project, set up to produce a biopesticide for locust control in Africa, set guidelines for germination on production of conidia to be >90% immediately after mass production. The yield from mass production is also an important economic factor to consider. In order to make biopesticides competitive with their chemical counterparts, Wright *et al.* (2001) stated that the ability to economically produce at a dose rate of 1×10^{13} conidia per hectare would be required to stay cost competitive with chemical insecticides. Finally, particle size needs to be considered as part of the quality control procedure, as this is related to the application equipment used to apply the agent and the droplet size produced. Ideally for spraying, particle sizes need to be <100 μ m to pass through spray equipment nozzles.

Throughout the developmental stages of this project, conidia were mass-produced by Sylvan – Somycel and samples were sent to CABI for quality control checking. The methods and results are presented below.

Materials and methods

Contamination checks

Approximately 0.1 g of conidia were added to 10 ml of sterile 0.05% Tween 80 using aseptic technique. The suspension was vortex-mixed thoroughly to suspend conidia and then sonicated for 3 min to break up any chains of conidia. A dilution series was then prepared from this stock solution by transferring 1 ml of stock into 9 ml of sterile 0.05% Tween 80 using a sterile pipette. The -1 dilution was then mixed thoroughly and 1 ml of this was transferred to another glass vial containing 9 ml of sterile 0.05% Tween 80. This process was repeated until a dilution series to -8 was prepared. Contamination plates were prepared by pipetting 200 μ l of each suspension from the dilution series onto two 90 mm Petri dishes containing SDA+TW (Sabouraud Dextrose Agar plus Tap Water). The suspension was then evenly dispersed over the plate using a sterile glass spreader. Plates were incubated at 25°C for 3 days before contamination was assessed. An assessment of contamination involved counting the number of colony forming units (CFU) of *B. bassiana* and the number of CFU of contaminants.

Conidia per gram

Conidia per gram were counted using either the -2 or -3 suspension from the contamination check. A small amount of the suspension was transferred to a haemocytometer slide using a Pasteur pipette and the numbers of conidia were counted. From this count, the conidia per gram could be assessed.

Viability

A small amount of conidia were suspended in 9ml Shellsol T and the suspension was sonicated for 3 min to break up any chains of conidia. 1-2 drops of suspension were spread across a 3 x 50mm SDA+DW (Sabouraud Dextrose Agar plus Distilled Water) Petri dishes. Plates were incubated at 25°C for 24 h, before they were removed and assessed for germination. Plates were read at x 200 magnification and at least 300 conidia were scored as germinated or un-germinated. A germinating conidium was characterised by the growth of a germ tube equal or longer in length than the conidium itself.

Moisture content

A HG-53 Mettler Toledo Moisture Analyzer machine was used to assess the moisture content of conidia. Approximately 0.5 g of conidia were weighed out and put into the machine, which was run on mode 5 at a drying temperature of 110°C. The moisture content was noted from the printout produced by the machine.

Particle size analysis

For some of the samples, particle size analysis was undertaken using a Helos Particle Size Analyser (laser diffraction; Sympatec GmbH). Each sample was run through the machine three times (apart from IMI 386243, batch 019 which was tested twice). Results display the volume median diameter (VMD) of particle size.

Results

Sylvan - Somycel Samples – Quality Control Assessment on Samples of *Beauveria bassiana*, IMI 389521

In general, the quality of mass-produced samples from Sylvan - Somycel was very high (Table 15). On only one occasion was contamination detected from a sample from a mass production run. Viability was excellent (>90%) for samples 1, 2, 6, 7, 11, 12 and 13. Moisture content varied and samples 2-9 showed low moisture content results, which is good for long-term storage prospects. Those samples above 5-6% in general would need to be dried further to ensure good storage. Conidia yields were in general excellent, showing significant improvement from initial studies

carried out at CABI (the highest conidia per gram achieved before Sylvan mass production scale up was 2.3×10^9 /g).

Sylvan – Somycel Samples – Quality Control Assessment on Samples of *Beauveria bassiana*, IMI 386243

Again, for isolate IMI 386243, results were generally very good. No contamination was detected in the mass produced conidia and moisture contents were reasonable. Viability was lower than ideal in sample 017R and 017E (Table 16). Conidia yields were excellent for this isolate throughout the different mass production runs, which was a significant improvement on initial mass production results from CABI, which produced 5.8×10^8 /g for this isolate.

Table 15. Quality control results for samples of *Beauveria bassiana*, IMI 389521. M.C. = Moisture content. * unknown batch number received 21/11/06 ** calculated from CFU count.. Samples 024 and 025 were not tested for moisture content. Samples are listed in date order of being received, with 13 being the most recent.

No.	Sample	M.C.	Conidia/g powder	Germination	Contamination
1	IMI 389521*	7.3%	2.90×10^{10}	95.8 %	No
2	521-003	5.33 %	4.90×10^{10}	92.0 %	Yes
3	521-004	5.25 %	9.09×10^9	69.5 %	No
4	521-005	5.91 %	6.69×10^9	68.6 %	No
5	521-006	5.86 %	7.12×10^9	73.4 %	No
6	521-007	3.70 %	2.21×10^9	91.2 %	No
7	521-009	4.31 %	4.64×10^9	90.4 %	No
8	521-021	4.77 %	3.97×10^{10}	78.9 %	No
9	521-022	5.50 %	4.19×10^{10}	82.6 %	No
10	521-023	9.85 %	5.40×10^{10}	78.8 %	No
11	521-024	-	$8.9 \times 10^{10**}$	91.1%	No
12	521-025	-	3.45×10^{10}	95.5%	No
13	521-026	8.36%	9.1×10^{10}	90.6%	No

Table 16. Quality control results for samples of *Beauveria bassiana*, IMI 386243

No.	Sample	M.C.	Conidia/g powder	Germination	Contamination
1	243-017R	4.76 %	4.30×10^{10}	55.6 %	No
2	243-017E	5.39 %	5.37×10^{10}	85.1 %	No
3	243-018	6.60%	1.85×10^{10}	90%	No
4	243-019	5.70%	4.36×10^{10}	91%	No

Particle Size Distribution

Figure 34 gives a comparison of the VMD (volume median diameter) of samples produced using different methodologies sent from Sylvan-Somycel. Table 17 gives an overview of the range of average particle sizes recorded for each sample of IMI 389521. Ideal particle sizes for spraying are <100µm, therefore, sample 003 and 007 appear to have the best VMD and particle size. When examining the largest particle sizes (99%) it appears that sample 007 is the only production method which has 99% of particle sizes less than 100µm. Table 18 shows the particle size results for two batches of IMI 386243. For both batches the largest particles are > 200 µm, which could present problems when formulating the conidia to spray as opposed to apply as a dust.

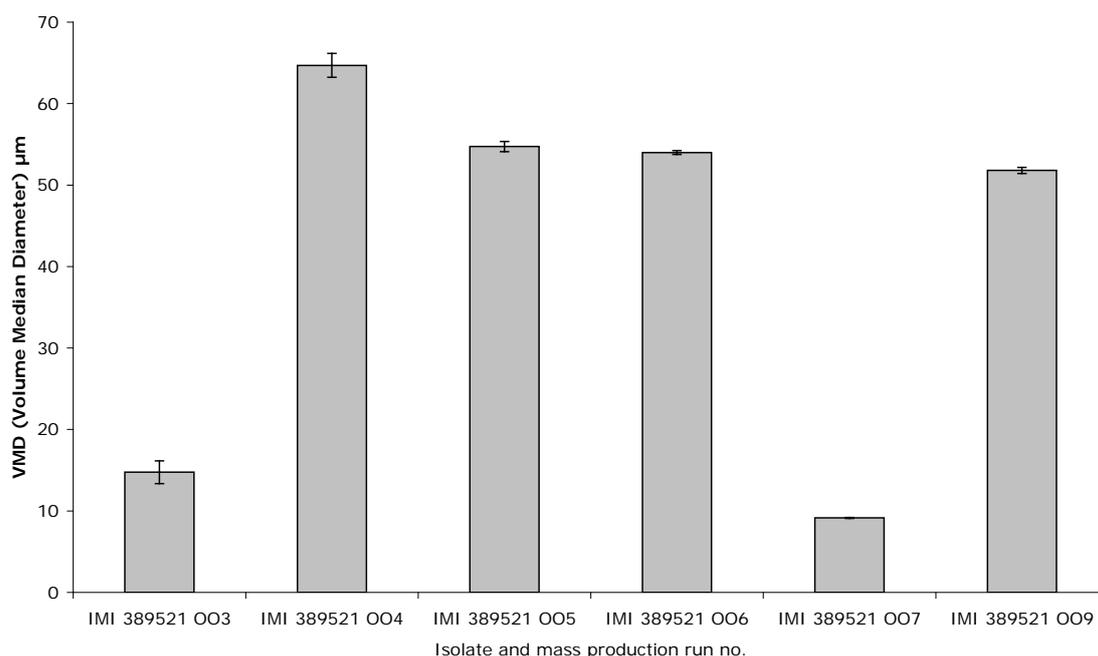


Figure 34. VMD (volume median diameter) of conidia from mass production runs of IMI 389521 (± 1 SE calculated from 3 reps through particle analyser for each sample).

Table 17. Particle size results for selected IMI 389521 samples.

Batch No.	Particle size (µm) at percentage distribution			
	99%	90%	50%	10%
003	191.1	37.3	2.1	0.8
004	206.8	145.2	50.5	5.9
005	162.4	112.1	49.6	5.4
006	153.6	107.1	49.2	8.2
007	75.4	26.2	2.8	0.9
009	160.4	112.0	45.3	2.7

Table 18. Particle size results for selected IMI 386243 samples.

Batch No.	Particle size (μm) at percentage distribution		
	90%	50%	10%
018	239.4317	51.498	3.683
019	219.238	47.282	2.874

Discussion

The quality of mass-produced conidia by Sylvan-Somycel throughout the project has been very high and the yield has increased with further refinements to the mass production technology, since initial laboratory scale mass production experiments were conducted. Moisture content has been generally good, but certain samples had conidia with higher moisture content than required and would have benefited from further drying. According to Hong *et al.* (2001), the storage time of conidia of *B. bassiana* can be increased by drying to levels below 5% and similar guidelines are recommended for conidia of *Metarhizium anisopliae* produced for locust control during the LUBILOSA programme.

The particle sizes of IMI 389521 need to be reduced in general for good particle suspension in oil formulations and effective spraying. Ideally, droplet sizes for fine oil sprays are 50-100 μm (Bateman and Chapple, 2001). For dust formulations, particle size is less of an issue for application; however, particles <10 μm increase the risk of inhalation by the operator (Bateman and Chapple, 2001). The method of application has an important bearing on the ideal size of particle for application. Viability of conidia for IMI 389521 and IMI 386243 from recent mass production runs has been excellent.

Safety

Introduction

The entomopathogenic fungus *B. bassiana* Vuillemin was first described around 170 years ago and, since then, has been widely researched for its potential to control insects. *B. bassiana* is widely distributed throughout the world from temperate to tropical areas, from deserts to bogs (Zimmerman, 2007). Although *Beauveria* conidia have been isolated from the air, they are not a common airborne contaminant and are more commonly found in the soils or on diseased insects (Zimmerman, 2007). It has been reported that *B. bassiana* has been isolated from over 700 species of insects (Goettel *et al.*, 1990).

Recent research with mycopesticides has produced significant improvements in their efficacy and opened up new areas for use. Obviously, the use of fungal biocontrol agents in close proximity to foodstuffs or livestock raises legitimate concerns as to safety.

General safety considerations

The growing interest in the exploitation of micro-organisms as biological control agents in agriculture has raised concerns about the safety of these organisms. Consequently, there is a need for a risk assessment to determine the level of hazard involved and an appreciation that any intervention in an ecosystem will have an impact. Compared with chemical pesticides, mycoinsecticides have features that provide ecologically sound pest control. They are selective to varying degrees, often suitable for integrated management techniques, may provide an extended period of control by remaining within the environment (or even establishing permanently), are biodegradable (Goettel and Johnson, 1992) and fundamentally safe (Copping, 1998). Frequently, the carriers may present more of a problem than the biological component, but even this is likely to be minor especially with the introduction of the new European Union regulation concerning the Regulation, Evaluation, Authorisation and restriction of CHemicals (REACH) that came into force on the 1st June 2007 (www.hse.gov.uk/reach). The most likely detrimental effect of a mycoinsecticide is that host depletion reduces the populations of natural enemies such as parasitoids and predators (Goettel *et al.*, 1990).

The hazards posed by mycoinsecticides to non-target organisms fall into the groups of toxicity, allergy and direct infection (Austwick 1980). The first two involve direct threats to humans and other vertebrates, especially domestic animals, while the third case theoretically relates to any organism. In this review, allergy and direct infection will not be discussed in detail. Allergy problems could occur in manufacturing plants producing vast quantities of dry particles. Appropriate containment and protective clothing removes the risk. At application, formulations and protective clothing provide adequate protection. Direct infection of mammals with *B. bassiana* is extremely unlikely with any isolate as body temperatures exceed the level at which the fungus can grow. Isolates used for biological control will be selected on many features, but if an isolate was found that grew above 37°C it would not be used in a product. This leaves the issue of metabolic production.

Metabolite production

There have been a few very good recent reviews of the safety of *Beauveria* species, in particular Strasser *et al.* (2000a, b), Vey *et al.* (2001) and most recently Zimmermann (2007). These papers examine the evidence of the safety of using mycopesticides, and especially *B. brongniartii*, in the environment. Although many *Beauveria* isolates can be shown to produce metabolites (Table 19) (and many cannot be shown to produce metabolites (Zimmermann, 2007)), this is usually in

laboratory conditions with nutrient rich media and there is often marked variation in levels and type of metabolites produced according to isolate (Strasser *et al.*, 2000b). Particular care was taken in this project to avoid choosing isolates that showed signs of metabolite production (i.e. pigment production) when mass-produced. Strasser *et al.* (2000a,b) showed the production of oosporein by *B. brongniartii* in laboratory conditions to be quite high but when used in the field the levels were considerably lower (Table 20). Furthermore, the larvae were cockchafer larvae (*Melolontha melolontha*) which are considerably larger than storage pests. Hence, it is reasonable to expect that the metabolite production, if any, in storage pest insects will be less.

Table 19. Major metabolites produced by *Beauveria* species. Adapted from Zimmermann (2007).

Beauveria sp.	Metabolite	Reference
B. bassiana	Beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein	Strasser <i>et al.</i> (2000b); Vey <i>et al.</i> (2001)
	Oxalic acid	Roberts (1981)
	Bassiacridin	Quesada-Moraga & Vey (2002)
B. brongniartii	Oosporein	Strasser <i>et al.</i> (2000b); Vey <i>et al.</i> (2001)
	Oxalic acid	Roberts (1981)

Table 20. *In vitro*, *in vivo* and *in situ* production of oosporein by *B. brongniartii*. Adapted from Strasser *et al.* (2000a; 2000b).

Substrate	Maximum amount of oosporein
Batch reactor	270 mg/l
Solid substrate (barley kernels)	2.0-3.2 mg/kg
Mycosed larvae	0.23 mg/larvae
Plant biomass	Not detected
Potato tubers	Not detected
Soil + BCA	0.02 mg/m ²
Soil + mycosed larvae	6.4 mg/m ²

Fungal metabolites have been shown to be toxic in some cases. For example, oosporein is toxic to 1-day old male chicks with an LD₅₀ = 6 mg/kg (Vey *et al.*, 2001). Also, mice and hamsters had a LD₅₀ = 0.5 mg oosporein/kg body weight when injected intraperitoneally but, when given a daily oral dose of 7 mg oosporein/kg body weight to mice over 47 days, there was no lethal effect (Vey *et al.*, 2001). Although beauverian has been shown to be toxic to brine shrimps, *Artemia salina*, (LD₅₀ = 2.8 µg/ml water), neither beauverian nor bassianolide has been shown to exhibit mammalian or plant toxicity. Furthermore, trials failed to detect oosporein in potato tubers even using instrumentation that could detect levels as low as 2.4 mg oosporein/kg potato tubers (Seger

et al. (2005). These examples demonstrate that in realistic situations (i.e. when not injected with neat oosporein), it is highly unlikely that *Beauveria* metabolites will be harmful to the environment, humans or other vertebrates.

One of the most relevant recent works on risks from metabolites is that from an EU funded project on Risk Assessment of Fungal Biocontrol Agents (www.rafbca.com), which had the general objective of identifying metabolites produced by fungal biocontrol agents and to establish whether they enter the food chain and if they pose a risk to human and animal health. This project concluded that metabolites from biopesticides do not enter the food chain and that the use of fungal biocontrol agents used in Europe and their relevant metabolites do not harm humans or the environment. The amount of metabolites from a formulated product or mycosed insects will be relatively small (Strasser *et al.*, 2000b) and they may represent a small fraction of that found naturally in soils.

Summary

Fungal biocontrol products using *Beauveria* species have a long history of use and have been well tested but there have been no serious detrimental cases reported (Zimmermann, 2007). Strasser *et al.* (2000b) point out that *Beauveria brongniartii* has been registered according to EU Directive 91/414/EEC, showing that this fungus and its metabolites are not a risk to human and animal health. Any potential fungal biocontrol agent will have to undergo similar testing before it can be released on the market, ensuring that all safety issues are covered. However, all the evidence to date indicates that metabolites produced by *Beauveria bassiana* show no risk to humans, animals or the environment.

3.4.3. Step 3.3 Risk assessment of potential non-target effects

Introduction

The commercial success of a microorganism to control pests and diseases of stored grain requires no side effects of the treatment on the grain. In trials by Harper & Lynch (1981) and Lynch *et al.* (1981), it was found that microorganisms can prevent germination of barley seeds and the growth of young barley seedlings by providing a sink for available oxygen and producing metabolites that were phytotoxic. This study investigated an entomopathogenic fungus of interest as a biological control agent for stored grain insect pests and its effect on barley seed germination when used as a seed coat. The former scenario anticipates very little contamination of seed from treated premises and the latter was tested to evaluate very high doses.

Materials and methods

Trial 1: barley seeds were mixed with dry conidial powder of *B. bassiana* (IMI 389521) for 2 min in a glass beaker; ca 100 g of seed was mixed with 0, 0.01, 0.1, 0.5 and 1 g conidia (1.8×10^{11} viable

conidia/ g). A Petri dish (9 cm diameter) was lined with filter paper and 25 treated barley seeds placed inside. The filter paper was moistened with 10 ml tap water, covered and left at 25 ± 2 °C for 48 h, after which time the number of germinated seeds was assessed. There were two dishes per treatment and the trial was carried out eight times. Trial 2: barley seeds were treated with conidia as above then 100 seeds placed into a Petri dish (9 cm diameter) lined with filter paper and moistened with 4 ml tap water. The seeds were left at 16 ± 2 °C for 48 h then seed germination assessed. There was one dish per treatment and the trial was carried out three times. Trial 3: barley seeds (ca 100 g) were mixed for 2 min with 0 and 1 g viable conidia and 1 g of heat-killed conidia. A Petri dish (9 cm diam.) was lined with filter paper and 25 treated barley seeds placed inside. The filter paper was moistened with 10 ml tap water, covered and left at 25 ± 2 °C for 48 h and the number of germinated seeds assessed. There were two dishes per treatment and the trial was carried out five times.

Data analysis

In all experiments, the results from the repeat trials were similar and were, therefore, combined. Any % data was arcsine-transformed before significance testing (Dytham, 2003) (the data presented in the paper is pre-transformed data). Analysis of variance with appropriate factors was used to analyse treatment effects in all trials, with Tukey's test and linear regression used to analyse differences and relationships between treatments (Genstat 11th Edition, VSNI). Differences between treatment means were considered significant at $P < 0.05$.

Results

Trial 1: at 25 seeds/ dish with 10 ml water the volume of conidia had a significant effect on barley seed germination ($F = 47.9$; $df = 4,35$; $P < 0.05$) (Figure 35); seed germination increased as conidia volume increased ($y = 45.3 + 28.2x$; $r^2 = 0.73$), rising from around 50 % at 0, 0.01 and 0.1 g conidia up to ≥ 84 % at 0.5 and 1.0 g conidia. Trial 2: at 100 seeds/ dish with 4 ml water the volume of conidia had a significant effect on seed germination ($F = 46.1$; $df = 4,10$; $P < 0.05$) (Figure 36); seed germination decreased as conidia volume increased ($y = 70.1 - 25.3x$; $r^2 = 0.90$), falling from > 80 % at 0 g conidia to 50 % at 1.0 g conidia. There was no significant reduction between 0-0.1 g conidia/100 seeds. This was a realistic dose. Trial 3: There was a significant effect of conidia presence vs absence on seed germination ($F = 63.4$; $df = 2,12$; $P < 0.05$) (Figure 37); seed germination was 50 % with no conidia, 86 % with viable conidia and 74 % with non-viable conidia.

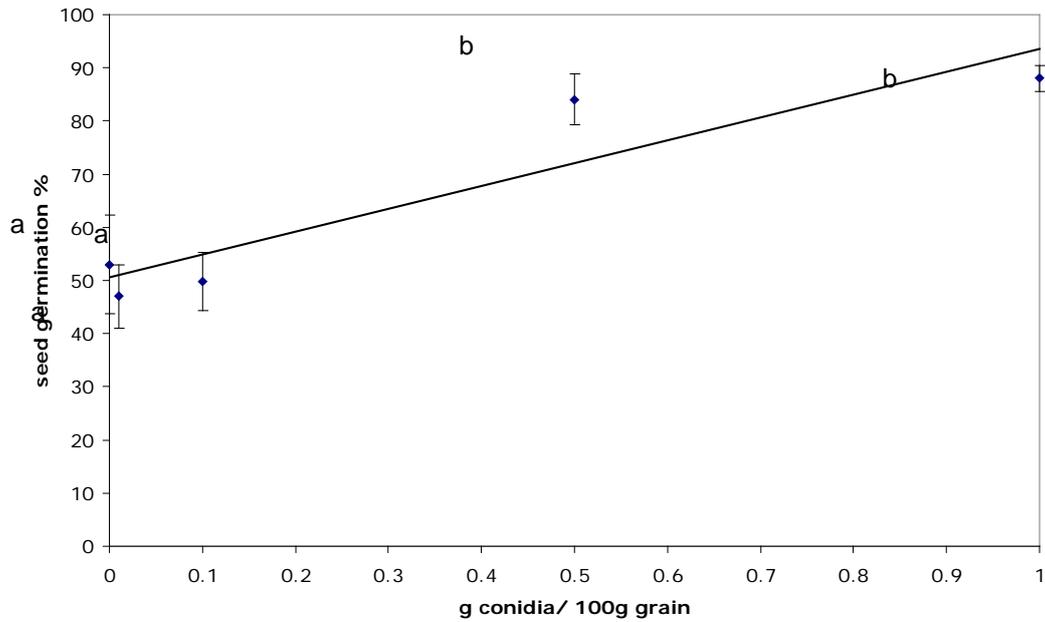


Figure 35. Trial 1: Mean germination of barley seeds following inoculation with *Beauveria bassiana* (IMI 389521). Seeds treated with: 0, 0.01, 0.1, 0.5 and 1.0 g conidia (1.8×10^{11} conidia/ g)/ 100 g seed. Germination of seeds assessed after 48 h at 25 °C; 25 seeds per dish with 10 ml tap water. Means sharing the same letter are not significantly different ($P > 0.05$).

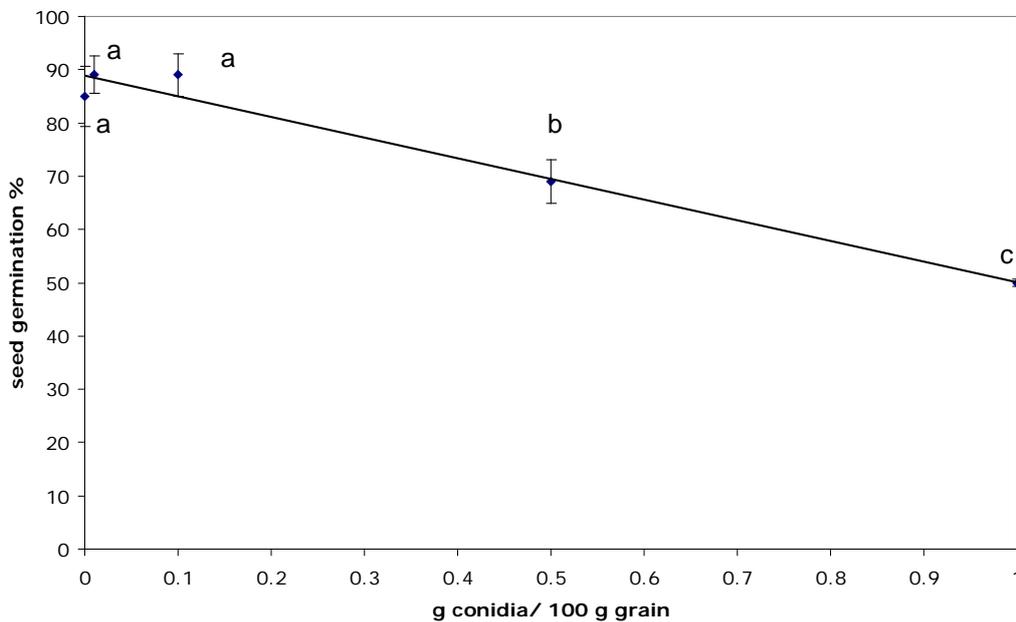


Figure 36. Trial 2: Mean germination of barley seeds following inoculation with *Beauveria bassiana* (IMI 389521). Seeds treated with: 0, 0.01, 0.1, 0.5 and 1.0 g conidia (1.8×10^{11} conidia/ g)/ 100 g seed. Germination of seeds assessed after 48 h at 16 °C; 100 seeds per dish with 4 ml tap water. Means sharing the same letter are not significantly different ($P > 0.05$).

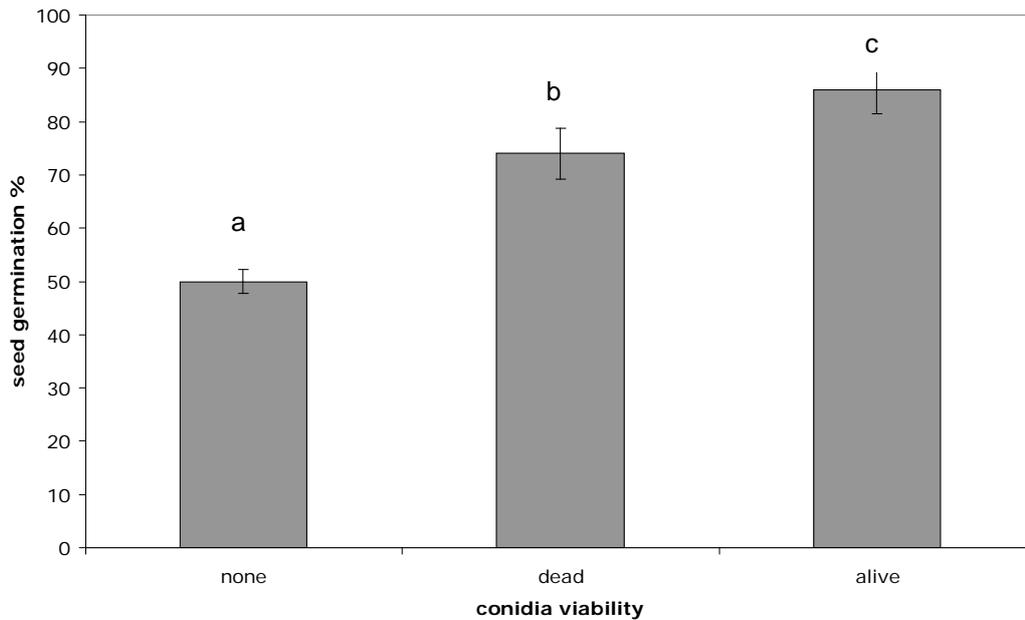


Figure 37. Trial 3: Mean germination of barley seeds following inoculation with *Beauveria bassiana* (IMI 389521). Seeds treated with a) no conidia, b) 1.0 g heat-killed conidia/ 100 g seed and c) 1.0 g viable conidia/ 100 g seed (concentration of conidia 1.8×10^{11} conidia/g). Seed germination assessed after 48 h at 25 °C. Means sharing the same letter are not significantly different ($P > 0.05$).

Discussion

When developing a crop protection product, it is important to examine side effects on the host plant of both the active ingredient and components in the formulation. The present study investigated the effect on seed germination of applying dry conidia as a seed-coat on barley seeds. Barley seeds are sensitive to environmental stresses, including excessive water (leading to imbibition injury), oxygen depletion and high temperature (Hosnedl & Honsová, 2002). In the absence of conidia, barley seeds in the present trial germinated best at a combination of the lower temperature (16 °C) and the lower moisture availability of Trial 2. When presented with the higher levels of moisture (a combination of fewer seeds and higher levels of added water) and temperature (25 °C) of Trials 1 and 3, seeds were relatively slow to germinate, only 50 % had germinated after 48 h. Adding a seed-coating of the *B. bassiana* fungus increased seed germination under the more temperature and water stressed conditions of Trials 1 and 3; this may have been a combination of conidia providing an absorbent, protective barrier which reduced the rate of imbibition and the germination of the conidia, which may have stimulated seed germination directly by the production of beneficial secondary metabolites or indirectly by out-competing inhibitory microorganisms on the surface of the seed (the seeds used in the trial were unsterilised). Results from Trial 3 in particular, using both dead and live conidia, enhances the idea that there was a combined protective and stimulative effect of the seed-coat. However, in Trial 2 it was observed that at the more favourable seed germination conditions of 16 °C and lower moisture levels, a seed-coating of the fungus had an

inhibitory effect on seed germination. Inhibitory thresholds for both lowering temperatures and O₂ availability may have been breached due in part to the germinating conidia on the seed surface.

The results indicated that a seed-coat treatment of dry *B. bassiana* could affect the timing and/or ability of barley seeds to germinate, and should be investigated further. However, at the levels of *B. bassiana* contamination that could occur on barley in treated premises, no harmful effects were noted.

3.5. Objective 4. Acceptability and practical use

Use of the biopesticide will depend upon its acceptability to all interested parties and proof that it will function effectively under conditions that are found in typical storage premises. These two aspects were addressed in the following four steps. **Step 4.1:** The biopesticide approach needs to meet the requirements of users and be acceptable to the public. From the start of the project, factors that might affect uptake by industry were identified. This included learning what industry users would consider an acceptable level of control. **Step 4.2:** It is known that the different constructional materials used in storage premises can influence the efficacy of conventional insecticides (Cox *et al.*, 2000) and the ability of a prototype formulation of *B. bassiana* to function (Cox *et al.*, 2004). Laboratory tests were undertaken on individual surfaces to ensure that the novel formulation developed in this project functioned effectively, regardless of surface type **Step 4.3:** The findings of the laboratory studies of efficacy were validated under conditions which bring together all the difficulties that have to be overcome in real situations, *i.e.* the need to function on combinations of different types of surface under challenging conditions of temperature and humidity, and deal with real infestations of target insects which may try to avoid treatment. **Step 4.4:** The versatility of the biopesticide for users, and its acceptability to the potential manufacturer and distributor were assessed.

3.5.1. Step 4.1 Requirements of users, the supply chain and consumers

Introduction

The development of any novel control method should consider not only the technical obstacles and developments, but also needs to consider how such a product would be viewed by stakeholders when used under 'real-world' conditions. In terms of the development of a biopesticide for the structural treatment of grain stores, the stakeholders can be divided in three groups:

- Likely users of the biopesticide product (application).
- Those who handle grain previously stored in biopesticide treated buildings.
- Consumers of products containing processed grain previously stored in biopesticide treated buildings.

The aim of this step was to identify the likely issues, if any, that will inhibit take up of a commercial product based on the biopesticide developed within the project. This was achieved by meeting with representatives of each stakeholder group to outline the project aims and progress, to ascertain any concerns and to identify information that ideally the project would need to provide. The information was fed-back to the Project Management Group to inform and better deliver the programme of work.

Methods

Representative organisations from each of the three key stakeholder groups were identified (Table 21). Meetings were arranged with a minimum of one organisation from each group. At each meeting the rationale for the project was explained and the findings from LK0914 (Mycopest) were presented. The key objectives for the current project were explained and the practical issues for the introduction and application of a biopesticide product were explored. In particular, the following points were covered:

- What factors of efficacy, ease of use and ease of adoption into existing storage hygiene protocols would influence the choice of control agent? In particular, what level of control would they regard as acceptable?
- Contracts. Current commercial grain contracts often stipulate zero contamination with living or dead grain-store insect pests. If biopesticides have lower efficacy, how will user representatives approach the requirement to change contracts?
- The protocols for using biopesticides are likely to be different from those of chemical control agents. How would these impact on storage practices? For example:
 - different application methods
 - longer period required after application/before grain loading
 - Understanding that users will need to be aware of possible efficacy effects related to humidity and temperature

Results

Consultations were held with The National Farmers Union, Agricultural Industries Confederation, The National Association of British and Irish Millers, The Maltsters Association of Great Britain and The Soil Association. The main concerns expressed by the majority of stakeholders fell into the following categories:

Table 21. Examples of representative organisations from three stakeholder groups

Users

National Farmers Union – pesticide and cereals groups

LEAF

Corporate farming world – Coop Farming Group

Supply chain participants

Assured British Food (owners of the ACCS mark)

UKASTA

British Retail Consortium

Grain Merchants

National Association of British and Irish Millers

Maltsters Association of Great Britain

Exporters

Retailers

Consumers

Consumers Association

Soil Association

Foods Standards Agency

1. Efficacy

The concerns in this area focussed on the range of pests that could be controlled with this type of product, whether limitations were imposed by temperature and humidity conditions, the potential shelf life of the product and the length of the residual effect after treatment.

2. Health and safety for operators.

The type of formulation was regarded as a key issue with stakeholders expressing concerns over the use of dust formulations due to potential respiratory risks. This was stated to be one of the main constraints to the use of another novel method for grain store pest control, diatomaceous earths. The potential for the production of secondary metabolites was also a concern.

3. Risk of contamination

The key concern was whether there could be a build-up of inoculum and whether the inoculum could pass further along the processing chain, for example, affecting germination of malting barley or taint in final products.

4. Use

With regard to the possibility of contamination, questions were asked by some stakeholders as to what measures could be taken to remove the fungal isolate from surfaces. Cost was seen as a key issue for some stakeholders, whilst others would be willing to accept an increased cost (assuming acceptable efficacy) if this led to a substantial reduction or elimination of the use of organophosphate insecticides.

Written responses were prepared by the project team for some stakeholders in response to the issues raised and these are summarised below:

- Health and safety for operators. Discussions showed formulation would be a key issue and that products which resulted in dust during application or subsequent handling of grain would not be viewed favourably. A variety of formulations were examined during the project, both liquid and powder, in terms of the shelf-life and the efficacy against insects. However, it is possible that any powder formulations could be used in conjunction with enclosed dispensers. The project is examining use in empty stores only and, therefore, there would be no direct application to grain. In the latter stages of the project, the extent of carryover (if any) of the formulation to grain stored after treatment was examined (Step 4.3).
- The isolates that were used in the project were originally obtained from storage beetle pests found in UK grain stores. One concern was whether the fungal isolates being used produce any metabolites that would be of concern with respect to mammalian toxicity. It has been shown that some strains of *Beauveria bassiana* do produce metabolites under certain culture conditions. Commercially available products containing *B. bassiana* have undergone extensive tests to ensure safety, and any product resulting from this project would need similar testing as part of the registration process (Step 3.2). The most relevant recent work on risks from metabolites is that from an EU-funded project on Risk Assessment of Fungal Biocontrol Agents (www.rafbca.com), which has the general objective of identifying metabolites produced by fungal biocontrol agents and establishing whether they enter the food chain and if they pose a risk to human and animal health. This project concluded that metabolites from biopesticides do not enter the food chain and that the use of fungal biocontrol agents used in Europe and their relevant metabolites do not harm humans or the environment (Strasser 2004).
- It was also questioned whether there is likely to be interaction between the biopesticide and mycotoxin forming fungi, specifically *Penicillium verrucosum*. These fungi are very different and it is viewed that no interaction would be possible.
- The issue of removal of the fungi used from the store was also raised. It should be possible to remove the fungi using a mild disinfectant, should this become necessary. *B. bassiana* is a naturally occurring organism and the isolates used in this project were originally found in UK grain stores. All safety issues would be addressed during product registration.

Efficacy and application issues

- The principle questions raised in terms of efficacy focussed on the range of species that would be controlled and the effect of environmental conditions such as temperature and humidity. The efficacy of the fungi against a wide range of the principle storage beetles found in UK was examined as part of this project. We know that it is more effective against some species than others but it is possible that formulation will improve efficacy for all species. It is known that the fungi are only effective over a certain temperature range;

temperatures that are too high or too low reduce, and finally totally remove, any efficacy. Similarly, humidity is also key and a high humidity is generally required. The project examined these issues in detail to ensure that the fungi will remain effective over as wide a range of conditions as possible. Formulation is likely to be key in this regard.

- The shelf life of the biopesticide before application and the longevity of its pest control efficacy after application were also queried. The shelf life of a range of formulations at two temperatures, one representing average room temperature and the other if the product was stored in a fridge, have been studied. Results show that the formulations offer a long shelf life and it is envisaged that any product would have a shelf life of at least 6-12 months. It is envisaged that annual treatments would be required, if insects are found during monitoring, in a similar way to current chemical treatments. The intention is that storekeepers would be able to undertake the treatments themselves. There may be a residual effect from insects becoming infected from insects that have died as a result of treatment. This is termed secondary cycling.

Other issues

- Queries were also raised with regard to the build-up of inoculum on dead insects, the routes by which the biopesticide may pass through the processing chain, and possible effects on the fermentation process or the end product. It is unlikely that the presence of dead insects would add significantly to the amount of product present and normal hygiene practices should also remove these from the store. As the product is intended for use in empty stores only, it is considered that presence further down the processing chain would be minimal. However, this requires further investigation to ensure this is the case.

Discussion

The meetings held with the various stakeholder groups enabled the key areas of concern of the introduction and use of a biopesticides as a structural treatment in UK stores to be identified. Many of the concerns raised were similar for all of the groups. The information was used to guide the experimentation within the project and data has been generated that can specifically address some of the issues raised. Although powder formulations were not considered to be the most appropriate by end users, they were included within the development of the formulations, as it was important at this stage to determine whether *B. bassiana* could achieve effective levels of control. The use of powder formulations within enclosed dispensers (bait stations) has also been considered within the project, both in laboratory and pilot scale studies.

3.5.2. Step 4.2 Efficacy on different surface types

Introduction

In a typical UK grain store, there are likely to be several different types of surface that would need to be treated as part of a structural treatment, for example, concrete floors, metal bins or walls and wooden grain walling. It is important that efficacy of the biopesticide is retained on all surface types and, ideally, that the viability of the conidia is maintained to provide a degree of persistency. Chemical insecticides can show different levels of efficacy and persistency on different surface types and, generally, efficacy on concrete is poorer than on other surface types (Cox *et al.*, 2000). The viability and efficacy of *B. bassiana* IMI 389521 when applied to wood, concrete and metal was examined in the laboratory. Further studies of the viability and efficacy of the conidia on different surface types are reported under Step 4.3 Pilot scale trials of biopesticide formulations.

Materials and methods

Viability of conidia on realistic surfaces in laboratory conditions

Realistic surfaces were prepared using steel, plywood and concrete. The steel and plywood were cut into 10 x 10cm squares and concrete was prepared from a paving slab cut approximately to these sizes. A suspension of conidia IMI 389521 was prepared in light white mineral oil (Sigma) at a concentration of 6×10^7 conidia/ml. The suspension (0.25 ml) was pipetted onto one side of each of the realistic surfaces. Surfaces were then incubated at 25°C for 1, 4 and 13 days. At each sampling time, the appropriate surfaces were removed and conidia were harvested from the surface by washing with 20 ml of Shellsol T and agitating the surface with a cotton wool bud. From these suspensions, germination tests were prepared to assess the viability of the conidia.

Efficacy of conidia on realistic surfaces

Small squares of wood, steel and concrete (approximately 7 x 7 cm) were treated with either the dry conidia powder of *B. bassiana* IMI 389521 or the dry conidia powder mixed with Entostat (3:1) at a concentration of 1 g/m² dry conidia powder. Adult *O. surinamensis* (Tram) were confined to each surface using a metal ring sealed to the surface using Pritt[®] adhesive. The inner surface of the ring was coated with Fluon to prevent insect escape. There were twenty insects in each replicate and five replicates of each surface type. Insects were left in contact with the surface for 24 hours after which they were removed to recovery arenas formed from a metal ring (5 cm diameter) on a Whatman No. 1 filter paper (7 cm diameter). A small quantity of diet was provided and mortality was assessed 14 days after the start of the experiment. Insects were maintained at 20°C, 70% r.h. throughout the test.

Results

Viability of conidia on realistic surfaces in laboratory conditions

Germination of the oil formulated conidia remained over 70% for the duration of the study when applied to wood and metal (Figure 38). The viability dropped over time by around 10% for both surfaces. The conidia applied to concrete showed poor viability from day 1 and a drop in viability over time was also observed.

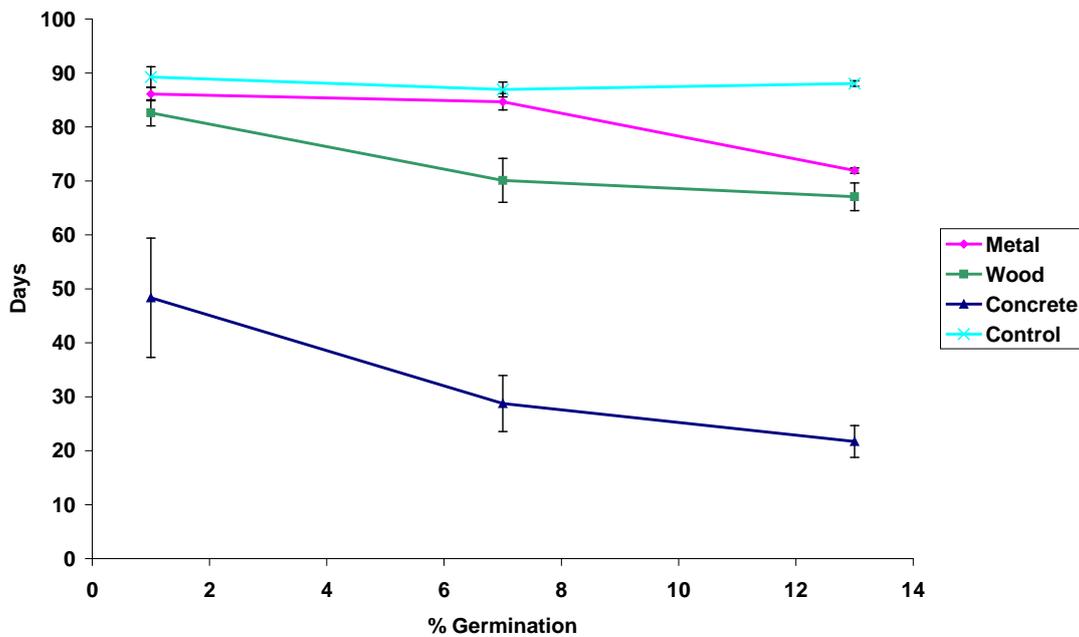


Figure 38. Results from laboratory scale experiments investigating the viability of conidia on realistic surfaces over 13 days.

Insect mortality after 14 days for the dry conidia powder + Entostat treatment exceeded that for the dry conidia powder alone on all three surface types (Figure 39). This difference was statistically different ($P < 0.05$) for the treatments on steel but not on concrete or wood. Mortality with the dry conidia powder + Entostat treatment was greater than 75% on all three surface types (Figure 39).

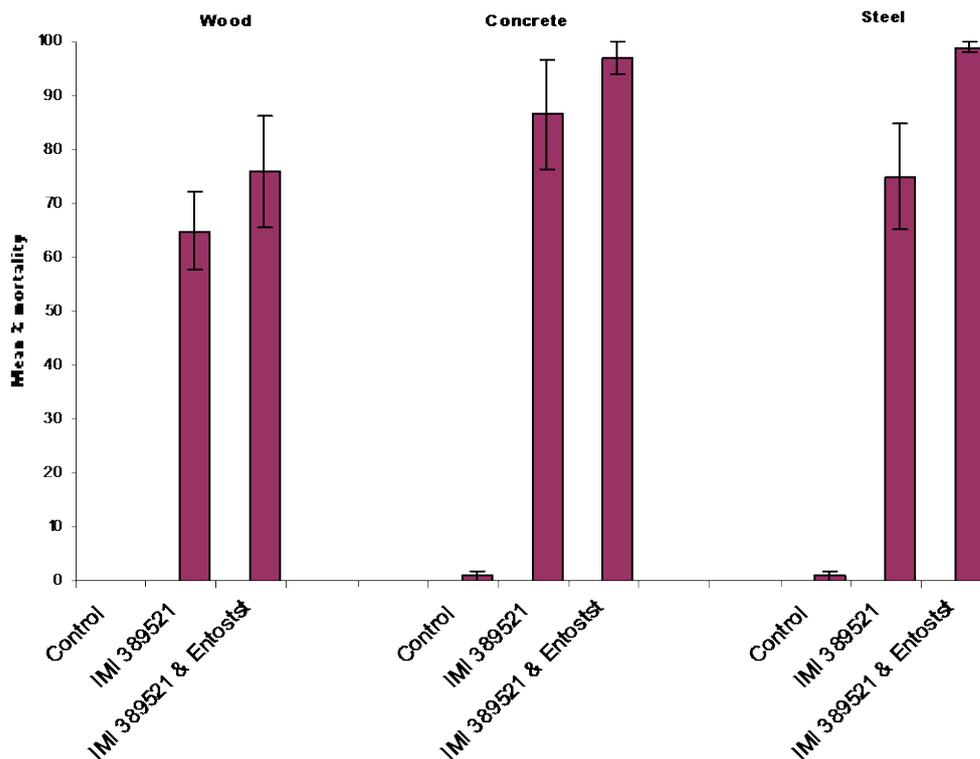


Figure 39. Mean (\pm SE) % mortality of *O. surinamensis* (Tram) 14 days after exposure for 24 hours to different surface types treated with IMI 389521 or IMI 389521 and Entostat.

Discussion

Viability of the fungus was found to be affected by the type of surface to which it was applied, with a substantial loss of viability observed on the concrete after only one day. The viability on wood and steel also decreased over the 13-day test period, but not to the same degree as the conidia on the concrete. Only one formulation type was tested in the laboratory and it is possible that other formulations may maintain the viability of the conidia to a greater degree. This was explored further in the pilot scale trials reported below.

The dry conidia powder, either alone or when mixed with Entostat, resulted in high levels of mortality of *O. surinamensis*. The mortality level on wood was slightly less than on steel or concrete. This may have been as a result of the surface of the plywood, which has small grooves in which the conidia can lodge, perhaps making contact with the insects more difficult. Persistency of the conidia on the various surface types was not explored in this laboratory trial, but the effectiveness of the treated surfaces over time was explored in the pilot scale trials (Step 4.3).

3.5.3. Step 4.3 Pilot scale trials of biopesticide formulations

Introduction

The laboratory studies have shown that isolate IMI 389521 has good efficacy against storage beetle pests under constant temperature and humidity conditions and that a good level of efficacy is maintained when the isolate is applied to different surface types. The conditions chosen for the laboratory studies were close to optimal for both the fungus and the insects. To act as an effective structural treatment in a grain store, it is important that efficacy is maintained for a reasonable period of time under the fluctuating environmental conditions that would typically be encountered in this situation. It was, therefore, important to test the fungus in a larger scale experiment under conditions that would typically be encountered in a UK grain store.

For the purpose of this experiment, two formulations, one oil based and one based on the dry conidia powder were chosen for further investigation. The primary aim of the pilot scale trial was to examine the efficacy of the two formulation types against three species of stored product beetle when applied to surface types likely to be found in a UK farm store. In addition, the viability of the fungus over time was assessed to provide information on the likely period for residual effect. Bidochka (2001) stated that, in order to understand the successes or failure of biological control agents, it is important to understand the disease epizootiology and the fungal microbial ecology. For this, the presence and persistence of the agent must be monitored in the treated area, including the coverage of the inoculum over the target area, the amount of inoculum applied (in context with the amount present prior to the trial) and the effectiveness for the spray to reach the target area. Various approaches can be used to provide this data. For example, spray distribution can be assessed by using fluorescent tracers such as Lumogen which can be detected using a UV light (Matthews, 1979) or alternatively oil sensitive paper can be used to capture the droplet deposition of oil formulations without the aid of UV light. To assess the amount of inoculum applied to the target area in terms of infective propagules, it is possible to monitor deposition using Petri dishes containing either agar or an appropriate liquid carrier, which are exposed at various time intervals during the trial (personal communication, Sharon Lawrence, CABI 2008). These are assessed by counting colony-forming units (CFU's) or using a haemocytometer. The various approaches were assessed in the pilot scale trial. Nansen *et al* (2007) stressed that, in order to control stored product beetles in grain stores, effective application to cracks and crevices needs to be sought. Therefore, an effective means of assessing the penetration of the spray into cracks and crevices was also examined in the pilot scale trial.

The results from the first pilot scale trial (see results) indicated that there were differences in the efficacy of the two formulations. However, the data also showed that the different application methods used resulted in a difference in the concentrations of the two formulations applied to the arenas. To establish whether both formulations should remain as candidates for further testing, it

was necessary to examine the effect of the two formulations when applied at similar concentrations. The second pilot scale trial examined the effect of the two formulations of IMI 389521 at two target concentrations (2.5×10^{10} conidia/m² and 5.1×10^{10} conidia/m²) on the mortality of three species of stored product beetle when applied to plywood arenas. In addition, the viability of the conidia in the two formulations under typical UK grain store conditions was again assessed. A comparison with a currently registered chemical pesticide, pirimiphos methyl, was also made.

Materials and methods

Fungal isolate

The *B. bassiana* isolate (IMI 389521) was produced by Somycel S.A. using the mass production method and was checked using in-house quality control (QC) procedures. A separate batch of conidia was produced for each trial. For the first trial QC results showed that there were 1.4×10^{11} conidia per gram, whilst for the second trial the isolate contained 7.6×10^{10} conidia/g. The isolates were sent to CABI Europe-UK Egham for further QC procedures including germination and contamination tests.

Formulations

Two fungal formulations were tested, one using a mineral oil carrier (a 50:50 mixture of light white mineral oil (Sigma-Aldrich, UK) and Shellsol T (Alcohols Ltd, UK)) and the other a dust formulation (conidia:Entostat 4:1) (Exosect Ltd). Formulations were made up immediately prior to treatment. Further details of the formulations and concentrations used in each trial are provided below.

Insects

Three species of insect were tested. These were *O. surinamensis* strain Tram (saw-toothed grain beetle), *Sitophilus granarius* strain Gainsborough (grain weevil) and *Cryptolestes ferrugineus* strain C124 (rust-red grain beetle). Insects used were of mixed age and sex. All three species were used simultaneously within the arenas. Insects were provided by the Invertebrate Supply Unit at Fera and were reared according to Fera Standard Operating Procedures (ISU/018, ISU/023, ISU/025, ISU/026, ISU/034).

Construction of arenas

The main arenas consisted of 2 m x 2 m plywood arenas with 0.5 m high walls (Figure 40). The plywood had not previously been treated with a fungicide. Insects were confined within the arenas within circular galvanized steel rings (approx 500 mm diameter, 150 mm high). The inside surface of the steel ring was coated with Fluon (Whitford Plastics, UK) to prevent escape of the insects. There were four rings in each arena. The rings were sealed to the floor of the wooden arena using decorators caulk so that insects could not get under the ring. A refuge made from a piece of

electrical conduit (25 x 16 x 200 mm), containing kibbled wheat to provide food for the insects, was placed in the centre of each ring, either immediately prior to treatment (Trial 1) or approximately 2 hours after introduction of the insects (Trial 2).

Monitoring of environmental conditions

Temperature was monitored throughout the trials using calibrated thermocouples (Type-T with a beaded tip and PTFE insulation (-50 to +250°C)) linked to a 'Squirrel' (model no.1045) temperature logger. Temperatures were logged every 60 minutes. The data was downloaded and analysed using 'SquirrelView' software. Temperature and humidity were also monitored using Tinytag dataloggers (TGP 1500, Gemini Dataloggers Ltd, UK) positioned in three of the arenas. Lights within the store were switched on during treatment and assessments but remained off at all other times.



Figure 40. Arena design –internal layout

Trial 1

The first trial was carried out within the Food and Environment Research Agency Grain Storage Facility and commenced in July 2008. The trial aimed to assess the two formulations of isolate IMI 389521 against the three species of insect when applied to arenas made of realistic surfaces, or held within a bait station, in the grain store environment. The trial examined the effect of direct application of the treatment to the insects or uptake of the treatment by the insects 24 h after application. Mortality of insects and on-going viability of fungus were assessed. In addition, assessments were made of the penetration of the oil formulation into crevices and the degree of contamination of the grain store during and after treatment.

Treatment of arenas

For the purposes of this trial, each plywood arena was divided in half and each half received a separate treatment. This enabled insects to be added to the two steel rings in each half of the arena either immediately prior to treatment or 24 h after treatment. The treatments used were:

- No treatment (control)
- Carrier oil only (carrier control)
- Oil formulation
- Dust formulation
- Dust formulation held within a bait station

The oil formulation was prepared by weighing out 5 g of conidia into a glass universal and adding a small amount of Shellsol T to prepare a paste. The volume of Shellsol T added was then made up to 25 ml and sonicated for 3 min. Immediately prior to the pilot scale trial, 25 ml of Light White Mineral Oil (Sigma) was added to the conidial suspension along with 3% lumogen (which acts as a tracer) and the suspension was thoroughly mixed. The oil formulation was applied to the arenas using an ULVA plus sprayer at a steady walking speed. The spray concentration in the tank was calculated to be 1.4×10^{12} on the basis of the conidia per gram and weight of conidia added. Deposition of droplets and levels of conidia applied were assessed using the methodology described below.

The powder formulation was a 4:1 mixture of conidia: Entostat (Exosect Ltd). Conidia (20 g) were weighed out and mixed thoroughly with Entostat (5 g), which contained 10% Globrite (a tracer) premixed. The dry powder formulation was applied in two ways. In the first method, a small duster (Mini Dust Applicator, Pest Free Homes, UK) was used to apply the powder at four cardinal points around the inner edge of the metal ring that would contain the insects. In the second method, a small quantity of the powder was placed in a bait station. Two types of bait station were used: a PC floor trap and an Exosect trap (Figure 41a). Both types of bait station were positioned in the steel rings assigned to this treatment (Figure 41b). Insects were able to enter and leave these traps and were therefore able to make contact with the conidia. The 'traps' also contained a small quantity of kibbled grain as a food source/attractant.

The amount of the dust formulation applied was assessed by weighing the applicator containing the dust formulation before and after applications were made to the 50 traps used in the pilot scale trial. An average amount per 'puff' was calculated and used to estimate the amount of conidia applied to the test areas.



A. B.

Figure 41. Bait stations used in Trial 1. A) An Exosect trap (left) and PC floor trap (right) showing dust treatment and kibbled wheat as a food source. B) the assembled bait stations *in situ*.

During the treatment period, the side of the arena that was not to receive treatment was covered with plastic sheeting. Floor areas in the vicinity of sprayed arenas were covered with plastic sheeting, which was disposed of in an appropriate manner in case of overspray. Arenas were positioned in a randomized design. The treatments for each arena are also randomized. There were four replicates for each treatment.

The effect of treatment on insect mortality

Insects were introduced to the steel rings approx. 2 hours before treatment (insects were therefore present during treatment) or 24 hours after treatment. All three species were present within each of the steel rings and 50 insects of each species were used in each replicate, except the oil carrier and oil formulation treatments where initially 75 insects were introduced. To ensure that the fungal isolate used had good efficacy against the insects, 25 insects of each species were removed from the oil carrier and oil formulation treatment replicates two hours after treatment. These insects were held in 90 mm Petri dishes with a small amount of kibbled wheat at 20°C, 70% r.h. in the laboratory. Insect mortality was assessed after 14 days.

Insect mortality in the steel rings was assessed after 7, 14, 21 and 42 days. A visual assessment with minimal disturbance was made at 7 days. For each of the other assessment times, insects were collected from the rings into glass tubes with the aid of an artist's paintbrush. Refuges were removed from the rings and placed in self-seal plastic bags. Insects were examined and the numbers of live and dead insects of each species within each steel ring and the refuge was recorded. Dead insects were surface sterilized with sodium hypochlorite solution, maintained in a damp environment and examined after 3-4 days for evidence of mycosis. Live insects were returned to the rings from which they were removed for re-assessment of mortality at the appropriate time point. The refuges were also returned to the rings from which they had been removed.

Viability of conidia on realistic surfaces

The viability of the conidia on three different surface types, metal, wood and concrete was assessed (Figure 42). The metal used was steel (0.6mm thick, B&Q Ltd, UK) and the wood was plywood (6mm thick, B&Q Ltd, UK). The metal and wood were cut into 10 x 10 cm squares. Concrete was prepared using a ready-mix pack (Hanson Instant, B&Q, UK), which was mixed with tap water and poured into 90 mm Petri dishes and allowed to dry over a number of days.

Surfaces were cleaned thoroughly with alcohol and placed within the arenas during the trial. Conidia were applied at the same time and using the same methods as the treatments to the arenas. Four replicate samples of each surface material were placed into each arena and there were four arenas for each of the application treatments.

After application, one of each surface material was removed from each of the arenas after 24 h, 5 d, 9 d and 42 d. Surfaces were washed using 10 ml of Shellsol T whilst agitating the surface with a cotton bud in order to remove any conidia present. Conidia suspended in Shellsol T were then stored at 5°C until germination tests could be set up. Previous work (not reported) has shown that germination is not affected when stored in Shellsol T at 5°C for this isolate. Germination tests were set up on 50 mm SDA (Sabouraud Dextrose Agar, Oxoid, UK) plus distilled water plates, using 1-2 drops of the Shellsol/conidia from each sample spread evenly over each plate. Three plates were set up per sample and were incubated at 25°C for 24 h. Germination was assessed by counting at least 300 conidia and marking them as germinated or non-germinated. A conidium was considered to be germinating when the germ tube emerging was longer than the conidium itself.

If the concentration of conidia was too low for assessment in germination tests, colony forming unit (CFU) counts were made. CFU counts were prepared by spreading 200 µl of the conidial suspension across 90mm SDA+TW plates and incubating at 25°C for 3 d or until visible colonies formed. Colonies were then counted and a note made of the numbers formed. If there was complete growth over the agar plate of fungus, this was termed as a lawn. If there was not a lawn, but over 300 colonies, a result of >300 was recorded. If the number of colonies were less than 300, then the actual figure was noted. The CFU method was unable to determine the number of non-viable conidia.

Viability was also assessed in terms of the efficacy against insects. Treated squares were removed from the arenas 7, 20 and 42 days after treatment and taken to the laboratory. Adult *O. surinamensis* (Tram) (20) were confined on each surface within a glass ring coated with Fluon. A small amount of kibbled wheat was added as a food source and the insects were kept at 20°C, 70% r.h. for 14 days at which point mortality was assessed. Additional 'traps' containing the dust

formulation were also treated and left in the arenas and then removed at the same time points. Each trap was placed within a small arena formed from a 20 cm diameter metal ring sealed to a Whatman No. 1 filter paper using Polyfilla to which twenty adult *O. surinamesis* were introduced. These insects were also kept at 20°C, 70% r.h. and mortality was assessed after 14 days. At each time point there were four replicates for each surface type with each treatment and four replicates with the Exosect trap and the PC floor trap.

Assessment of *B. bassiana* levels in the grain store

The levels of *B. bassiana* in the grain store were assessed using two separate methods (Figure 42). Levels of conidia were only assessed as part of the oil treatment. The first method used the exposure of selective agar to the environment to assess the numbers of CFU of *B. bassiana* found within the store. Selective agar, 30% SDA+CuCl₂ was prepared in 90mm plates using a recipe from Shimazu and Sato (1996), which was modified by the addition of antibiotics. Plates were exposed in each arena receiving the oil treatment 1 day prior to spraying (for 20 min), on the day of spraying (20 min), and on days 1, 2, and 7 days post spraying (for 20 min). The number of *B. bassiana* and non-*B. bassiana* colonies were counted at each time point.

The second method used 90 mm Petri dishes containing 3ml of Shellsol T to act as 'traps' for conidia in the environment. Plates were exposed in each of the oil + conidia treatment boxes for 18 h prior to spraying, 30 min before, 30 min during and 30 post spraying and for 18 h post spraying on day 1, 2 and 7. Haemocytometer counts were carried out to assess the number of conidia in each Petri dish (using a Brightline haemocytometer) and CFU plates were also set up to assess the number of viable conidia present.

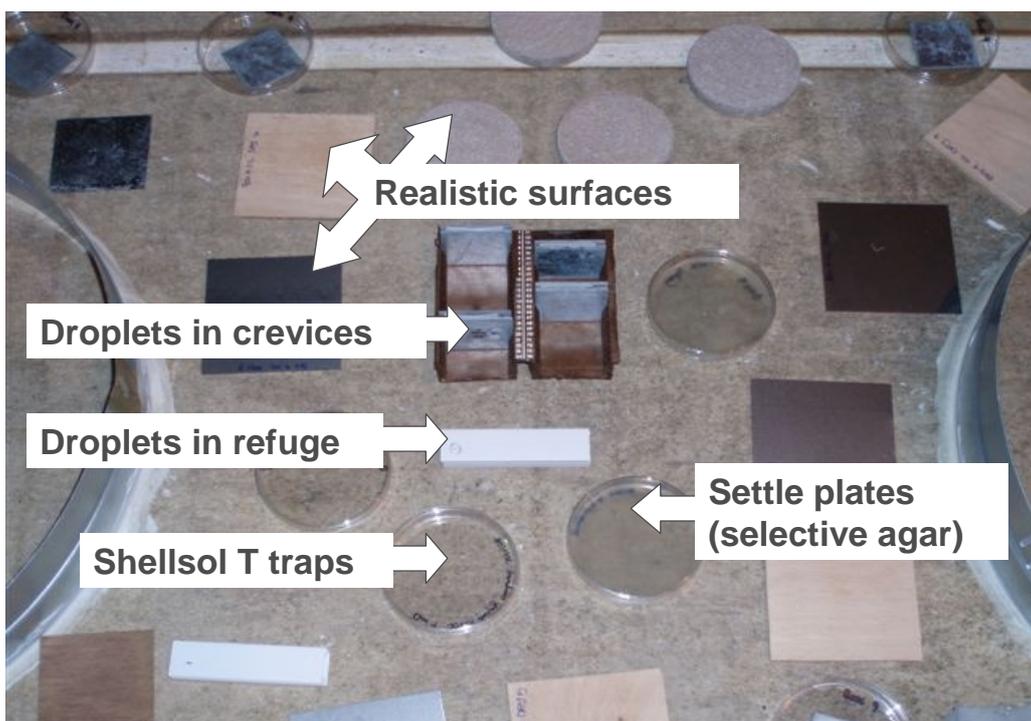


Figure 42. Methods of assessing viability of conidia, how many conidia have been deposited in a certain area and the ability of droplets to penetrate cracks and crevices

Spray coverage

To assess overall deposition of droplets in the target and non-target area, oil sensitive paper was laid both inside and outside the arena during the oil spray application. The number of droplets per $\frac{1}{4}$ cm² was then counted and from this the average number per m² was calculated. This methodology examined whether the droplets were drifting outside the experimental area.

Spray penetration

To assess the ability of the ULV spray to enter cracks and crevices, oil sensitive paper was placed in vertical and horizontal traps in order to assess the number of droplets entering (Figure 43a and b). Vertical traps were made up of two squares of metal held up in a rack with a piece of oil sensitive paper between. There were four replicates per arena and these traps were placed in four arenas. For the horizontal trap, the oil sensitive paper was placed in either end of the refuge containing food for the insects. Again, spray penetration was assessed by counting the number of droplets on the papers using the above methodology. One replicate was made per arena.

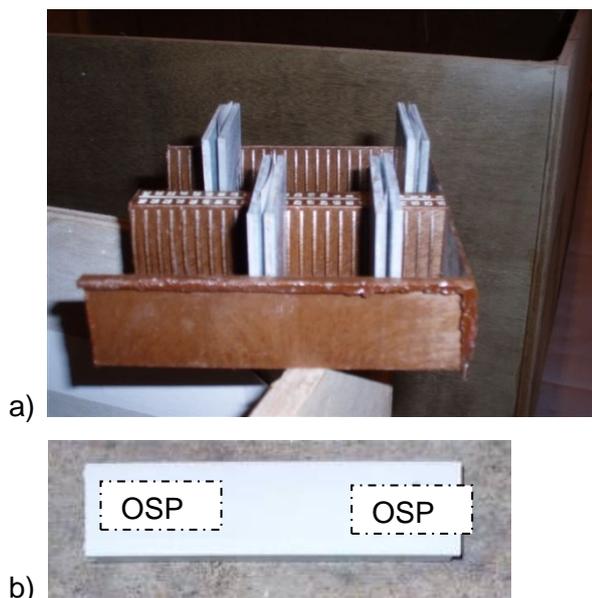


Figure 43. a) Oil sensitive paper between two metal plates allowed the vertical penetration of droplets to be assessed. b) Oil sensitive paper (OSP) was placed in both ends of the refuge and droplet penetration was assessed.

Trial 2

The second trial was carried out within the Food and Environment Research Agency Grain Storage Facility and commenced in June 2009. The trial compared the efficacy of the oil and dust formulations applied at two different concentrations (2.5×10^{10} conidia/m² and 5.1×10^{10}

conidia/m²). In addition, a comparison was made with a current recommended insecticide, pirimiphos methyl. Mortality of insects and ongoing viability of fungus were assessed.

Treatment of arenas

The arenas were cleaned approximately three months before use using Virkon at the recommended application rate. One week prior to the trial, arenas were swept and cleaned with water. For the purposes of this trial each plywood arena was divided into quarters with the steel ring in each quarter receiving a different treatment.

The treatments were as follows:

- No treatment (control)
- Mineral oil (oil control)
- Dust carrier (Entostat) (dust control)
- Water (insecticide control)
- Oil formulation 2.5×10^{10} conidia/m²
- Oil formulation 5.1×10^{10} conidia/m²
- Dust formulation 2.5×10^{10} conidia/m²
- Dust formulation 5.1×10^{10} conidia/m²
- Pirimiphos-methyl applied at the recommended rate

Treatments for each ring were randomly assigned to each arena with the single constraint that no treatment should be present in the same arena more than once. There were five replicate rings for each treatment. All metal rings were covered with cling film during the treatment period and the film was removed for the purpose of treating the ring. The dust formulation was weighed out and the appropriate amount was evenly distributed across the floor area of the assigned rings using a small brush. The oil formulation was applied using calibrated sprayers (Appendix 1). Three sprays were delivered within each ring from a height of approximately 50 cm. The pirimiphos-methyl and water treatments were applied in the laboratory. A known volume of pesticide solution or water was applied to the wooden squares using a hand held 'De Vilbis' paint sprayer. The spray gun was held approximately 20 cm above the surface of the square and the pesticide or water was sprayed onto the surface, working progressively across and down the square in zigzag movements, in order to obtain an even treatment across the surface. On an additional square, five filter papers were placed equidistant from each other and attached to the surface with a pin. The square was sprayed with the same volume of pesticide and method as that used for the test squares. The filter papers were subsequently analysed to determine the concentration of pesticide delivered.

After treatment, the squares treated with pirimiphos methyl were positioned in the large plywood arenas. The water treatment squares were positioned in the vicinity of the large plywood arenas.

The effect of treatment on insect mortality

Insects were released into the steel rings 24 hours after treatment. All three species were present within each of the steel rings and 50 insects of each species were used in each replicate. The refuge containing kibbled wheat was added to the arena approximately two hours after the introduction of the insects.

Insect mortality was assessed after 14 and 28 days. Insects were collected from the rings into glass tubes with the aid of an artist's paintbrush. Refuges were removed from the rings and placed in self-seal plastic bags. The numbers of live and dead insects of each species within each steel ring and the refuge was recorded. Dead insects were surface sterilized with sodium hypochlorite solution, maintained in a damp environment and examined after 3-4 days for evidence of mycosis. Live insects were returned to the rings from which they were removed after the 14-day assessment for re-assessment of mortality after 28 days. The refuges were also returned to the rings from which they had been removed.

Assessment of fungal viability

Small wooden squares (approx. 50 mm x 50 mm) were positioned in the arenas close to the rings and were treated at the same time as the arenas for insect assessment. These squares were removed 24 hours after treatment and after 15 and 29 days to coincide with the 14- and 28-day insect mortality assessments. Squares were collected and wrapped in aluminium foil and sent to CABI for assessment. Conidia were removed from the surface by rinsing with 10 ml Shellsol T. The surfaces were gently agitated with a cotton bud to ensure removal of conidia. The Shellsol T containing conidia was then further diluted to an appropriate concentration and germination tests were set up as described previously.

Statistical analysis

The difference in mortality between treatments using the biopesticide formulations and the respective controls/carriers was analysed by a generalized linear model (GLM) with a logit link function. Post-hoc tests were used to test for differences between the treatments by comparison of the least significant difference for the treatments. The data for the water control and the pirimiphos-methyl treatment in Trial 2 were analysed separately from the treatments and controls for the biopesticide. For these treatments, the percentage mortality was subjected to an arcsine transformation and treatments were compared using a two-sample t test.

Results

Trial 1

The average temperature recorded in the grain store throughout the trial was 14.9°C with a minimum of 9.1°C and a maximum of 23.5°C. There was very little variation in temperature observed between the ten arenas (Figure 44). The average humidity during the trial recorded using the Tinytag dataloggers was 83.2% with a minimum of 57.8% and a maximum of 95.3% (Figure 45).

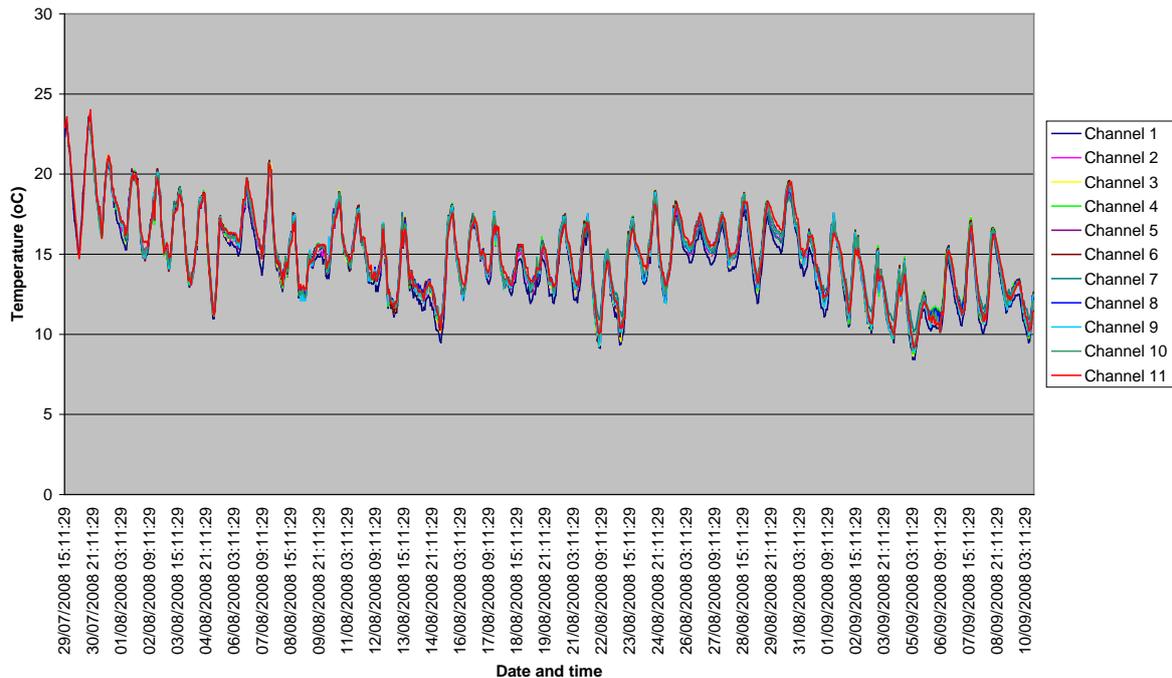


Figure 44. Temperatures recorded using the Squirrel datalogger in each of the ten arenas throughout Trial 1.

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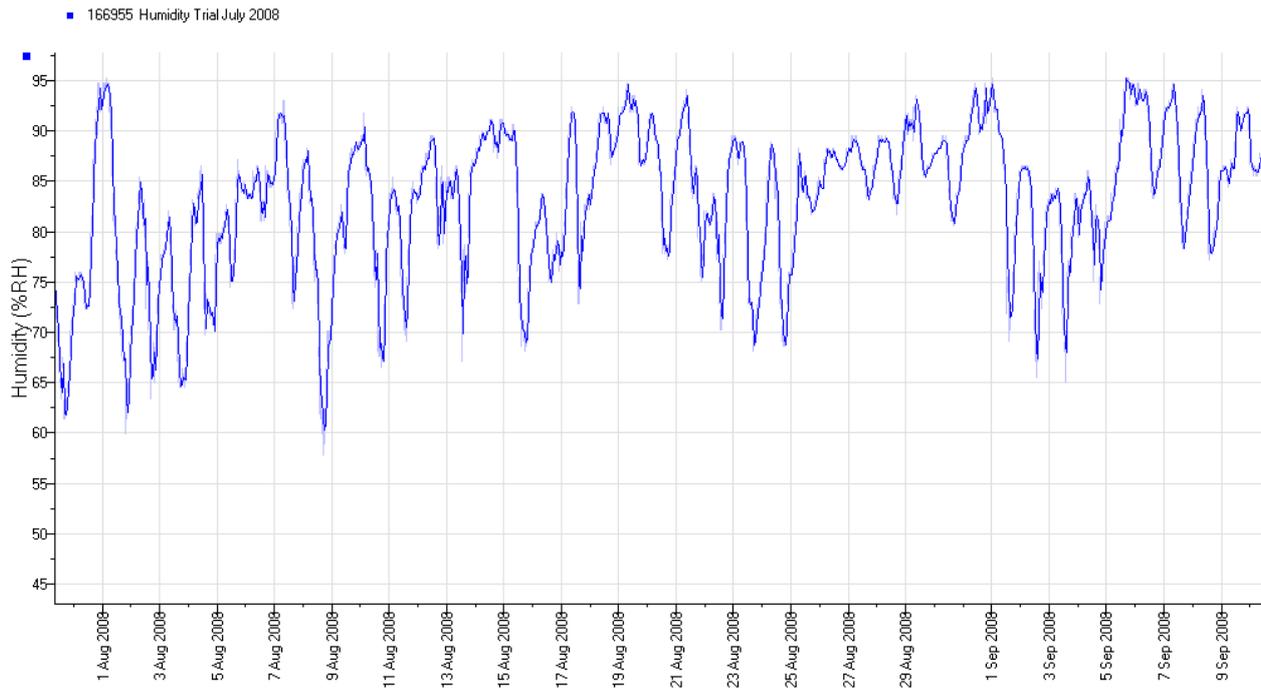


Figure 45. An example trace showing the humidity recorded within a plywood arena during Trial 1 from treatment to final assessment.

The exact quantity of the oil formulation delivered to each replicate could not be determined directly, but an indication was provided by the amount of conidia found in the shellsol 'traps'. This indicated that on average 1.1×10^8 conidia were applied per m^2 , which was below the target concentration. For the dust application, the weight of conidia applied to 50 traps was assessed and an average concentration of conidia applied was calculated. In total, 3.18 g of conidia were applied to 50 traps, therefore there were 0.0636g of conidia+Entostat per puff on average. It was assumed that the Entostat and conidia were mixed evenly; therefore the total weight of conidia applied was 2.544g. Over 50 traps this meant that on average 0.051g of conidia were applied per 'puff', which equates to 7.14×10^9 conidia per trap. For the application of powder to arenas, four puffs were made at each point of the compass in the arena, meaning that 2.86×10^{10} conidia (1.46×10^{11} conidia/ m^2) were applied to each arena.

The effect of treatment on insect mortality

A proportion of the insects that were released in the steel rings prior to the carrier oil and oil formulation treatments, and therefore were sprayed directly, were removed after 2 hours and returned to the laboratory. The insects were held at 20°C, a temperature at which the fungus will be more effective. Fourteen days after treatment the number of *S. granarius* sprayed with the oil formulation was not significantly different from the number treated with the carrier oil. The % mortality for both *O. surinamensis* and *C. ferrugineus* treated with the oil formulation was

significantly greater than for insects treated with the carrier oil (GLM $P=0.004$ and $P<0.001$ for *O. surinamensis* and *C. ferrugineus*, respectively) (Table 22)

Table 22. Derived mean % mortality of insects recovered from steel rings sprayed with the carrier oil or the oil formulation two hours after treatment and maintained at 20°C, 70% r.h. for 14 days. Figures in parentheses are the derived 95% confidence intervals.

	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Oil	9.0 a (4.2, 18.1)	5.0 a (1.2, 18.0)	9.3 a (4.7, 17.3)
Oil and conidia	11.8 a (6.0, 21.7)	53.0 b (37.6, 67.8)	81.2 +/- 2.1 b (71.7, 88.1)

In each column means followed by the same letter are not significantly different (GLM, $P>0.05$)

There was a highly significant effect of treatment with the biopesticide formulations on mortality for all three species, present in the rings at the time of treatment, after 14 days exposure (GLM, $F_{4,14}=53.11$, $P<0.001$, $F_{4,14}=82.64$, $P<0.001$ and $F_{4,14}=26.70$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively) (Table 23). Subsequent analysis showed that at the 5% probability level there were no significant differences between the mortality recorded in the rings with no treatment and those that had been treated with the carrier oil ($P>0.05$) (Table 23). Mortality of *O. surinamensis* and *C. ferrugineus* with the oil formulation containing conidia caused significantly greater mortality in comparison to the untreated rings ($P<0.05$) (Table 23). Mortality of all three species was significantly greater for the dust formulation both when applied to the surface and held within the bait stations ($P<0.05$) (Table 23). When allowance was made for multiple comparisons (Bonferroni adjustment, $P=0.005$) the differences between the dust formulation treatment and the bait station treatments compared with the control remained significant for all three species.

Table 23. Derived mean % mortality of insects 14 days after treatment. % mortality is expressed in terms of the number of insects recovered for each species. Figures in parentheses are the derived 95% confidence intervals.

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	0.5a (0.03, 7.9)	5.5a (2.8, 10.3)	6.7a (2.5, 16.5)
Oil carrier	0.7a (0.04, 10.5)	4.8a (2.2, 10.1)	14.7a (7.2, 27.7)

Oil formulation	11.0a (6.1, 19.0)	15.8b (10.9, 22.3)	52.8b (40.2, 65.1)
Dust formulation	67.0b (57.0, 75.7)	77.6c (69.1, 84.3)	97.1c (85.9, 99.4)
Bait stations	76.2b (66.4, 83.8)	86.1c (79.0, 91.0)	63.1b (50.1, 74.4)

In each column means followed by the same letter are not significantly different (GLM, $P>0.05$)

A highly significant effect of treatment with the biopesticide formulations on mortality for all three species present in the steel rings at the time of treatment was also found after 21 days and 42 days exposure (GLM, $F_{4,14}=41.76$, $P<0.001$, $F_{4,14}=56.73$, $P<0.001$ and $F_{4,14}=25.57$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively after 21 days and GLM, $F_{4,14}=71.27$, $P<0.001$, $F_{4,14}=86.44$, $P<0.001$ and $F_{4,14}=37.95$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus*, respectively after 42 days) (Tables 24 and 25). The efficacy of the oil formulation increased over time with significantly greater mortality compared with the control observed after 42 days exposure for all three species. Mortality also increased over time for the dust formulation, both applied to the surface and when present in the bait stations, and remained significantly greater than the control for all three species throughout the test period ($P<0.005$) (Tables 24 and 25).

Table 24. Derived mean % mortality of insects 21 days after treatment. % mortality is expressed in terms of the cumulative number of dead insects recovered after 14 and 21 days divided by the total of the number of insects recovered after 21 days and the number of dead insects after 14 days. Figures in parentheses are the derived 95% confidence intervals.

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	1.5a (0.2, 11.3)	8.6a,b (4.4, 16.0)	12.7a (6.2, 24.2)
Oil carrier	0.8a,b (0.02, 23.7)	6.5a (2.7, 14.6)	21.7a (11.6, 37.0)
Oil formulation	19.0b (11.2, 30.3)	20.4b (13.7, 29.2)	55.9b (43.3, 67.8)
Dust formulation	84.0c (72.0, 91.5)	87.1c (77.8, 92.9)	98.6c (85.8, 99.9)
Bait stations	92.7c (81.7, 97.3)	89.7c (81.3, 94.5)	81.4c (68.7, 89.7)

In each column means followed by the same letter are not significantly different (GLM, $P>0.05$)

Table 25. Derived mean % mortality of insects 42 days after treatment. % mortality is expressed in terms of the cumulative number of dead insects recovered after 21 days and 42 days divided by the total of the number of insects recovered after 42 days and the total number of dead insects after 21 days. Figures in parentheses are the derived 95% confidence intervals.

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	5.8a (2.5, 13.0)	11.7a (7.3, 18.1)	30.7a (22.3, 40.7)
Oil carrier	3.2a (0.7, 12.8)	11.6a (6.7, 19.3)	37.4a (27.2, 48.8)
Oil formulation	37.1b (27.6, 47.8)	31.7b (24.4, 40.0)	66.5b (57.4, 74.5)
Dust formulation	99.5c (91.6, 100)	95.2c (89.1, 97.9)	99.3c (91.8, 99.9)
Bait stations	97.2c (90.3, 99.2)	99.3c (93.8, 99.9)	95.3c (88.7, 98.1)

In each column means followed by the same letter are not significantly different (GLM, $P > 0.05$)

There was a highly significant effect of treatment with the biopesticide formulations on mortality for all three species, added to the rings 24 hours after treatment, after 14 days exposure (GLM, $F_{4,15}=35.80$, $P < 0.001$, $F_{4,15}=39.71$, $P < 0.001$ and $F_{4,15}=25.44$, $P < 0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively) (Table 26). Subsequent analysis showed that at the 5% probability level there were no significant differences between the mortality recorded in the rings with no treatment and those that had been treated with the carrier oil ($P > 0.05$) (Table 26). Mortality of *C. ferrugineus* with the oil formulation containing conidia caused significantly greater mortality in comparison to the untreated and oil treated rings ($P < 0.05$) (Table 26). Mortality of all three species was significantly greater for the dust formulation both when applied to the surface and held within the bait stations ($P < 0.05$) (Table 26). When allowance was made for multiple comparisons (Bonferroni adjustment, $P = 0.005$), the differences between the dust formulation treatment and the bait station treatments compared with the control remained significant for all three species.

Table 26. Derived mean % mortality of insects 14 days after exposure to surfaces treated 24 hours prior to introduction of the insects. % mortality is expressed in terms of the number of insects recovered for each species. Figures in parentheses are the derived 95% confidence intervals.

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	1.0 a (0.1, 8.7)	4.8 a (1.7, 12.9)	8.6 a (3.6, 19.3)
Oil carrier	4.5 a (1.6, 12.2)	4.6 a (1.5, 13.1)	13.1 a (6.5, 24.8)
Oil formulation	10.8 a (5.5, 20.0)	14.2 a (8.0, 24.0)	49.4 b (36.5, 62.3)
Dust formulation	72.5 b (61.4, 81.4)	74.1 b (61.8, 83.4)	93.1 c (82.1, 97.6)

Bait stations	56.8 b (46.1, 66.8)	84.8 b (73.8, 91.7)	64.5 b (52.6, 74.8)
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In each column means followed by the same letter are not significantly different (GLM, P>0.05)

A highly significant effect of treatment with the biopesticide formulations on mortality for all three species, added to the steel rings 24 h after treatment, was also found after 21 days and 42 days exposure (GLM, $F_{4,15}=34.13$, $P<0.001$, $F_{4,15}=44.36$, $P<0.001$ and $F_{4,15}=21.51$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively after 21 days and GLM, $F_{4,15}=46.09$, $P<0.001$, $F_{4,15}=54.70$, $P<0.001$ and $F_{4,15}=36.42$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively after 42 days) (Tables 27 and 28). The efficacy of the oil formulation increased over time with significantly greater mortality compared with the control observed after 21 days for *O. surinamensis* and *C. ferrugineus* and for all three species after 42 days exposure. Mortality also increased over time for the dust formulation, both applied to the surface and when present in the bait stations, and remained significantly greater than the control for all three species throughout the test period.

Table 27. Derived mean % mortality of insects 21 days after exposure to surfaces treated 24 hours prior to introduction of the insects. % mortality is expressed in terms of the cumulative number of dead insects recovered after 14 and 21 days divided by the total of the number of insects recovered after 21 days and the number of dead insects after 14 days. Figures in parentheses are the derived 95% confidence intervals.

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	1.0 a (0.06, 16.1)	6.7 a (2.7, 15.6)	12.5 a (5.3, 26.6)
Oil carrier	5.1 a (1.4, 17.0)	7.3 a (2.9, 16.8)	21.1 a (11.1, 36.3)
Oil formulation	24.3 a (13.9, 39.1)	24.2 b (15.8, 35.3)	62.0 b (46.4, 75.6)
Dust formulation	87.9 b (74.8, 94.7)	84.7 c (73.6, 91.6)	96.6 c (84.6, 99.3)
Bait stations	91.2 b (78.6, 96.7)	94.9 c (85.7, 98.3)	83.1 b,c (69.4, 91.4)

In each column means followed by the same letter are not significantly different (GLM, P>0.05)

Table 28. Derived mean % mortality of insects 42 days after exposure to surfaces treated 24 hours prior to introduction of the insects. % mortality is expressed in terms of the cumulative number of dead insects recovered after 21 days and 42 days divided by the total of the number of insects recovered after 42 days and the number of dead insects after 21 days. Figures in parentheses are the derived 95% confidence intervals.

Mean % mortality

	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	4.1 a (1.1, 13.3)	7.8 a (3.7, 15.8)	19.1 a (11.3, 30.4)
Oil carrier	9.6 a (4.2, 20.5)	10.7 a (5.6, 19.7)	45.1 b (32.1, 58.9)
Oil formulation	37.8 b (26.4, 50.8)	37.0 b (27.2, 47.9)	84.0 c (72.8, 91.1)
Dust formulation	96.5 c (87.6, 99.1)	90.9 c (81.8, 95.7)	99.4 c,d (89.8, 100)
Bait stations	94.4 c (84.9, 98.1)	98.7 c (90.6, 99.8)	98.8 d (90.6, 99.9)

In each column means followed by the same letter are not significantly different (GLM, $P > 0.05$)

Viability of conidia on realistic surfaces

Viability of the conidia applied to realistic surfaces in the dust treatment during the first trial remained over 80% for concrete and metal and just under 80% for the wood treatment over the first 5 days (Figure 46). At day 9, conidia from the concrete treatment displayed a drop in viability of almost 20% but retained this level of germination for the remainder of the 42 day trial period. Metal and wood showed good germination at day 9 but viability dropped by 27% for wood and 17% for metal between day 9 and day 42. Results for wood on day 42 had a high standard error and this was due to very few viable conidia observed in box 9 at this time point.

Results from the oil treatment were harder to obtain as fewer conidia were applied to each surface and, therefore, harvested. In some boxes especially on days 5 and 9, there were too few conidia to make an accurate assessment of germination, but where enough conidia were recovered, the results have been displayed in Figure 47. Over 9 days, germination on all treatments remained above 80% and only metal showed a drop in viability of about 10%. In order to gauge the viability of conidia over time, CFU counts were set up in order to confirm the presence or absence of viable conidia on the surfaces (Table 29). These counts demonstrated the presence of viable conidia after 42 days within the grain store environment.

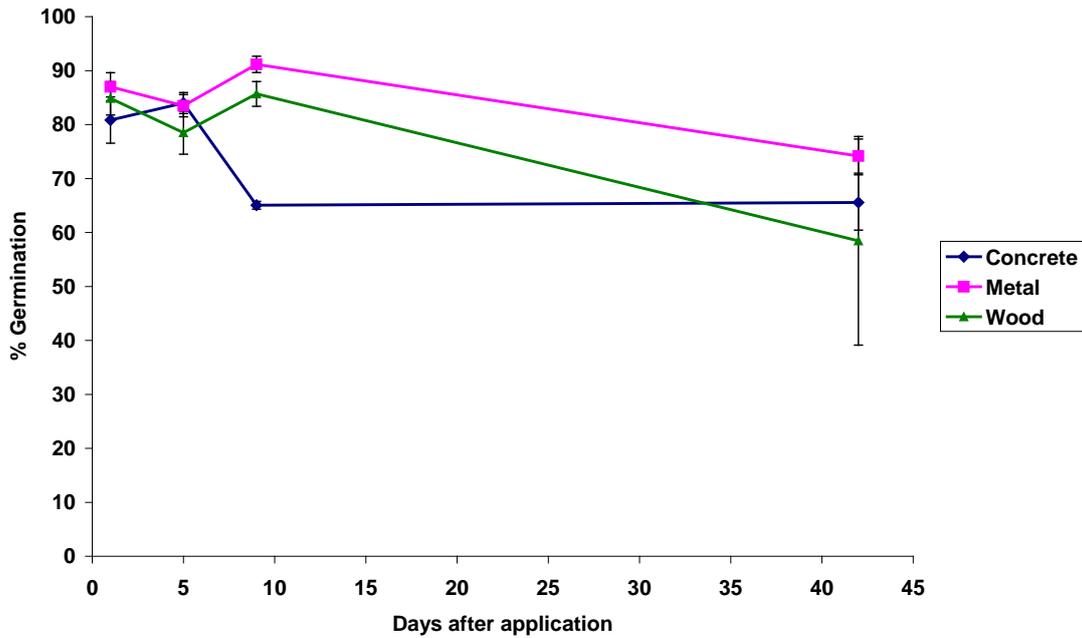


Figure 46. Viability of conidia applied as a dust treatment to realistic surfaces (steel, wood and concrete) over the duration of the pilot scale trial.

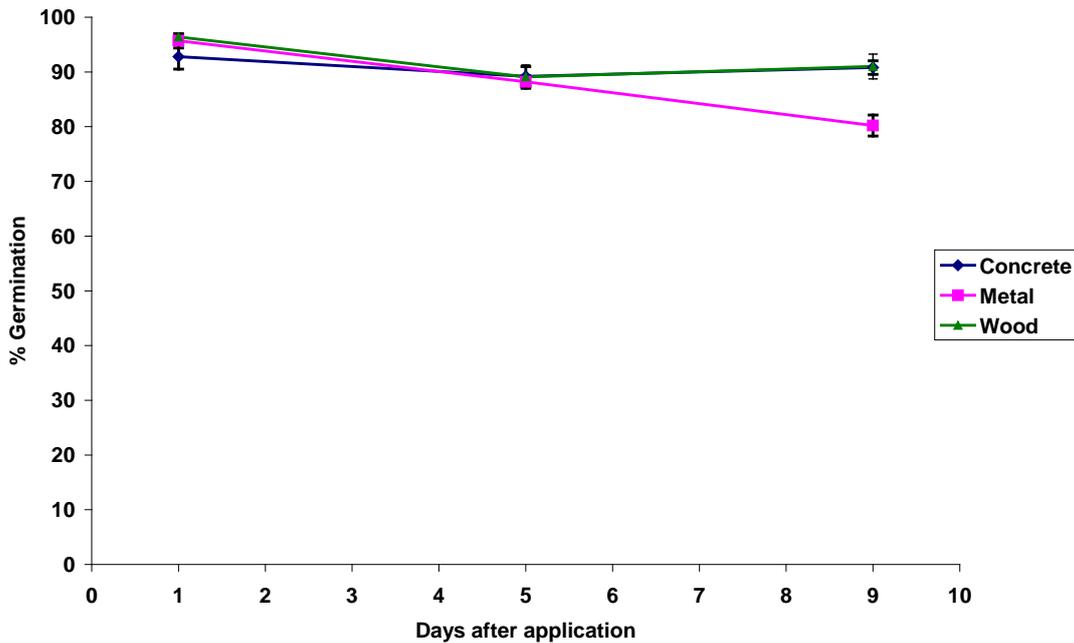


Figure 47. Viability of conidia applied as an oil treatment to realistic surfaces (steel, wood and concrete) over 9 days. Results could not be obtained from day 42 samples.

Table 29. CFU counts from the washings from oil treatment. Figures shown are the number of fungal colonies grown from samples harvested from realistic surfaces in each of the boxes over the duration of the trial. A lawn is characterised by being unable to distinguish individual colonies of the fungus and dense growth.

Box Number	Day 1	Day 5	Day 9	6 wks
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Concrete	10	>300	>300	>300	>300
Concrete	9	>300	104	>300	114
Concrete	7	Lawn	>300	73	>300
Concrete	5	>300	33	17	33
Metal	10	Lawn	10	Lawn	144
Metal	9	>300	36	Lawn	>300
Metal	7	>300	109	41	42
Metal	5	>300	57	Lawn	Lawn
Wood	10	>300	4	>300	28
Wood	9	Lawn	>300	133	54
Wood	7	Lawn	148	32	114
Wood	5	>300	>300	>300	>300

The viability of the conidia was also measured in terms of the efficacy on insects at three time points post-treatment. Surfaces and traps were removed from the grain store and insects were exposed to the surfaces in the laboratory. Seven days after treatment with the oil formulation, only insects exposed on the concrete surface showed any degree of mortality when compared with the controls (Figure 48). The dust formulation caused greater than 70% mortality on average for each of the three surface types (Figure 48). Mortality of insects in the arenas containing the PC trap with the dust formulation recovered 7 days after treatment was approximately 80% (Figure 48). Similar results were found after the surfaces and bait stations had been exposed in the grain store for 20 days (Figure 48). After exposure in the store for 42 days, the dust formulation in the PC floor trap was still very effective, resulting in 100 % mortality of the exposed insects (Figure 48). The efficacy of the dust formulation on the different surface types was lower than had been seen after 7 and 20 days but still resulted in mortalities greater than 50% (Figure 48). At all three time-points mortality was greater for the dust formulation applied to concrete than to steel or wood.

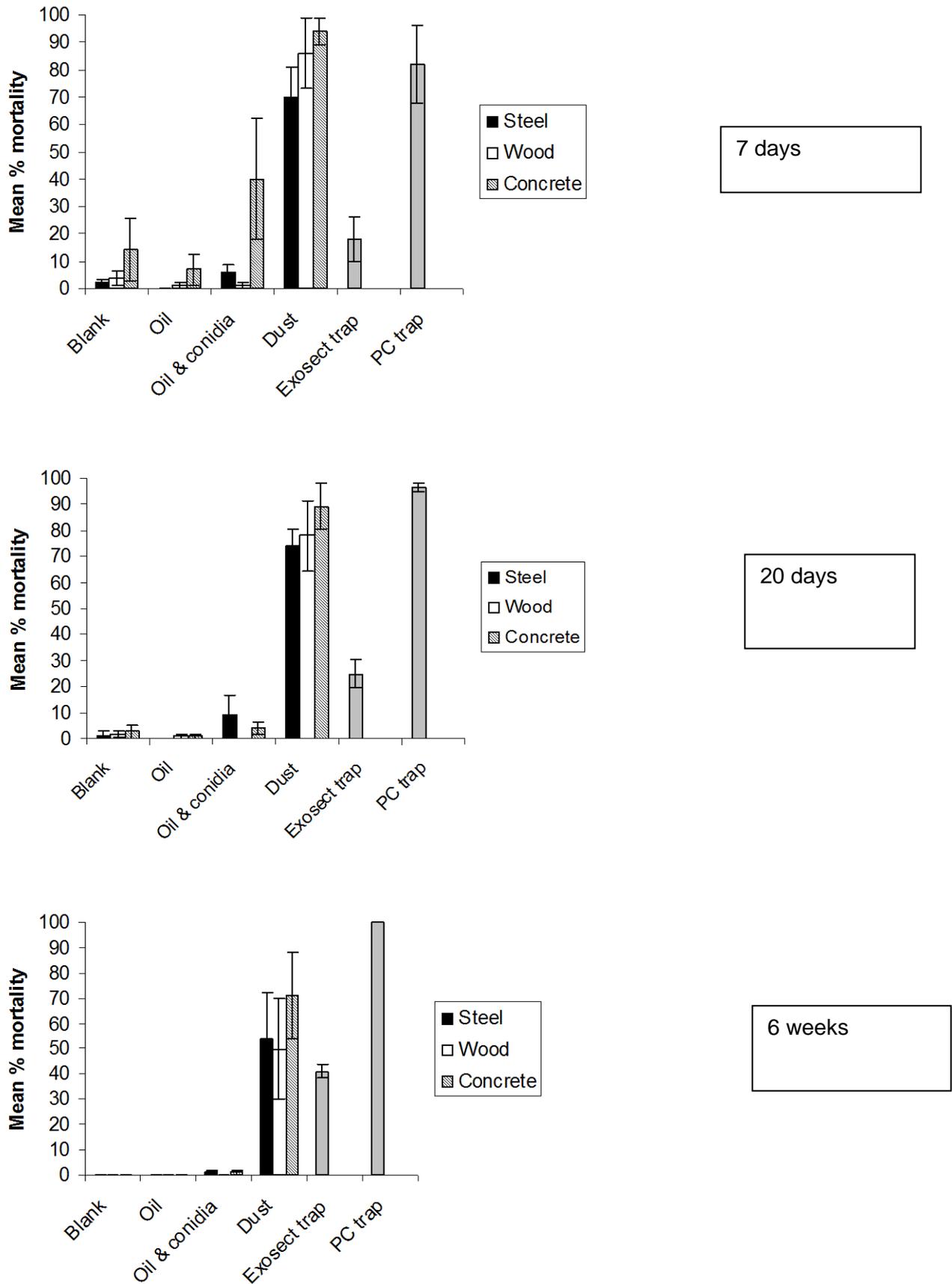


Figure 48. Mean (\pm se) % mortality after 14 days for *O. surinamensis* on surfaces or in bait stations removed from the grain store at three time points post-treatment

Amount of inoculum in the grain store prior to, during and post application of conidia

Prior to the application of the fungus to the arenas, selective agar plates were exposed to assess the level of background inoculum present (Table 30). No *B. bassiana* was found in any of the arenas prior to application. Levels increased dramatically at the onset of spraying, with a 'lawn' of fungus growing on the selective agar after incubation, from plates exposed during spraying. A lawn is characterised by being unable to distinguish individual colonies of the fungus and the growth is dense. The following days after application, the levels of *B. bassiana* detected were low, apart from box 9, 1 day after spraying. It is suspected that the increase in levels may have been due to a leak in the roof above the box, causing more airborne conidia. The results from the method using the shellsol 'traps' also showed a peak during spraying and then a drop back to very low levels (Figure 49). These results support those found on the selective plates. From this method, it was also possible to assess the level of airborne *B. bassiana* in comparison to background contaminants (Figure 50). It was found that levels of *B. bassiana* dropped to below those of the background contaminants two days after spraying.

Table 30. Number of colonies of *B. bassiana* found on selective agar exposed to the grain store throughout the field trial per m². Results were converted to per m² values from number of colonies per Petri dish.

	Days after application				
	<u>-1</u>	<u>0</u>	<u>1</u>	<u>2</u>	<u>7</u>
Box 5	0	Lawn	3.3 x10 ³	1.4 x10 ³	5.1 x10 ³
Box 7	0	Lawn	1.1 x10 ³	1.1 x10 ³	1.2 x10 ³
Box 9	0	Lawn	5.1 x10 ⁴	9.1 x10 ²	1.7 x10 ³
Box 10	0	Lawn	1.7 x10 ³	2.9 x10 ³	7.6 x10 ²
AVERAGE	0	Lawn	1.4 x10 ⁴	1.5 x10 ³	2.2 x10 ³

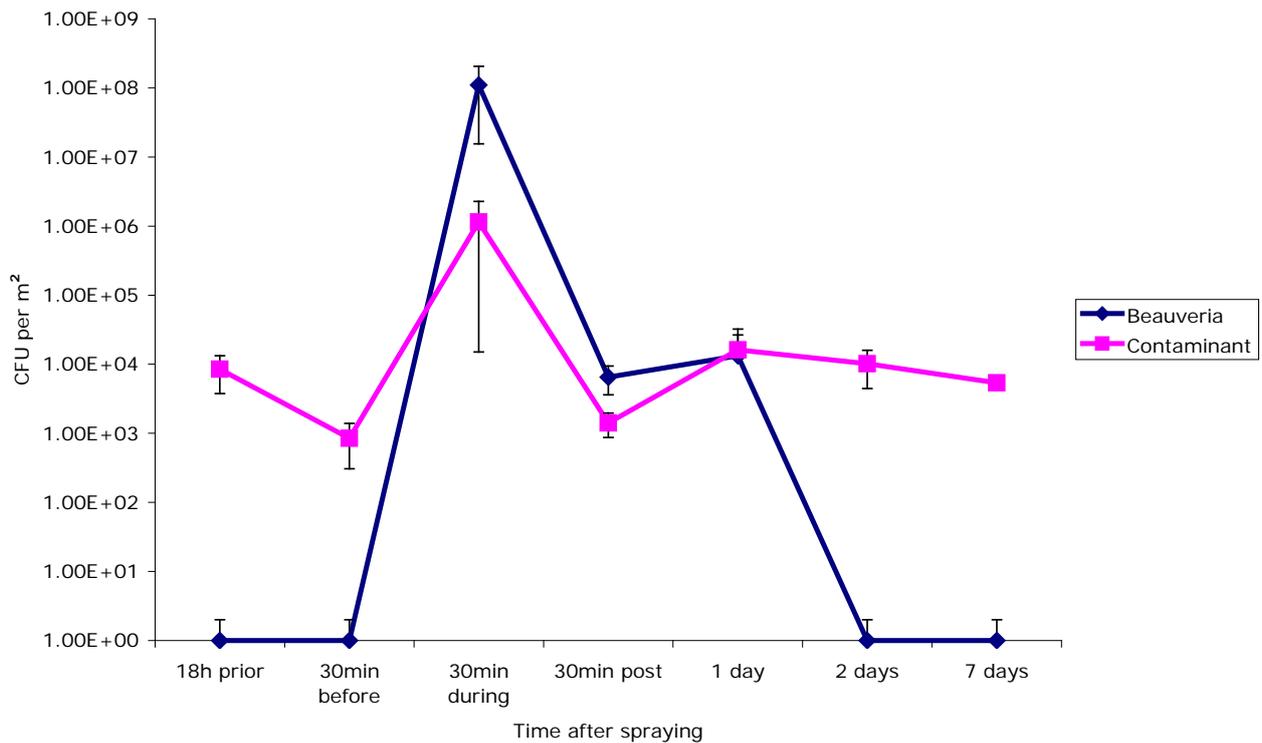


Figure 49. CFU's per m² throughout the course of the trial prepared from Shellsol T traps (grown on SDA+TW) (during numbers were obtained from -2 and -3 dilutions).

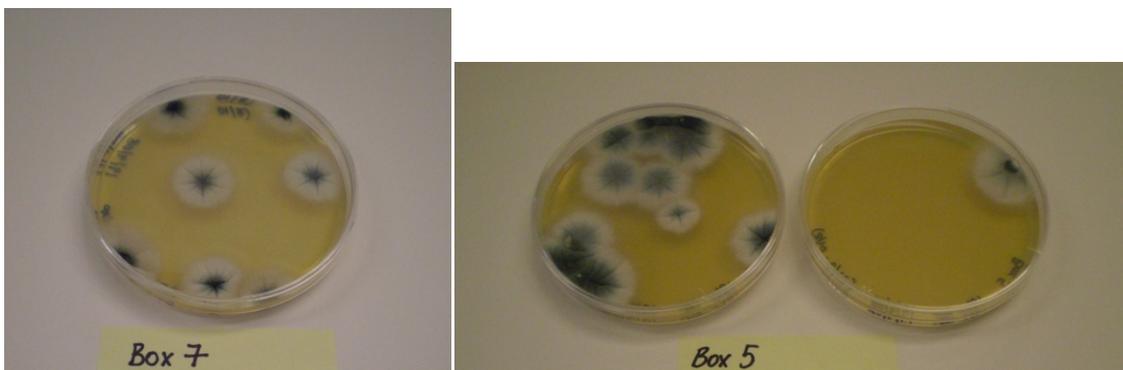


Figure 50. Example Petri dishes prepared from Shellsol T traps. No *Beauveria bassiana* colonies were found in these examples; however, background contaminants were suspected to be *Penicillium sp* or *Aspergillus sp*.

Spray coverage

The results from the assessment of the oil sensitive paper showed that droplets were landing in the targeted area for deposition. Significantly fewer droplets landed outside the arena indicating that the spray was correctly targeted (Tukey HSD, $p=0.03$). Photographs comparing the number of droplets deposited on the oil sensitive paper both within the arena and outside the arena are shown in Figure 51. The paper shows that the droplets are evenly spread across the target area indicating good application.

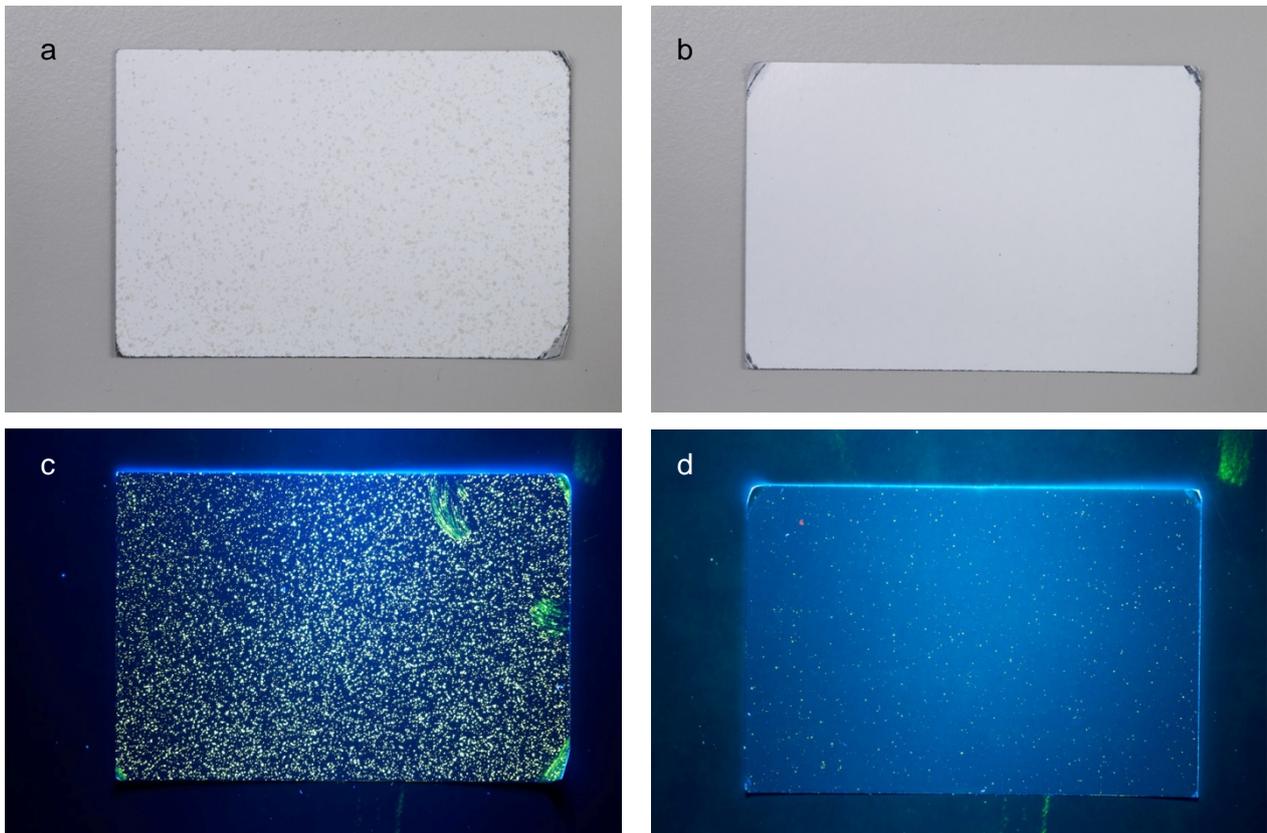


Figure 51. Spray deposition patterns during the trial. A (within arena) and B (outside arena) are on oil sensitive paper and C and D are the same papers illuminated by UV light to show the lumogen tracer.

Spray penetration

Spray penetration results showed that the spray when applied to a vertical crevice was able to penetrate and deposit, down to 4 cm (Figure 52). On the horizontal surface, it was found that spray was able to deposit up to 1 cm inside the 'crack' (Figure 53). Results appeared to show that droplets were more likely to be deposited at one end of the trap than the other, probably due to the direction of application.

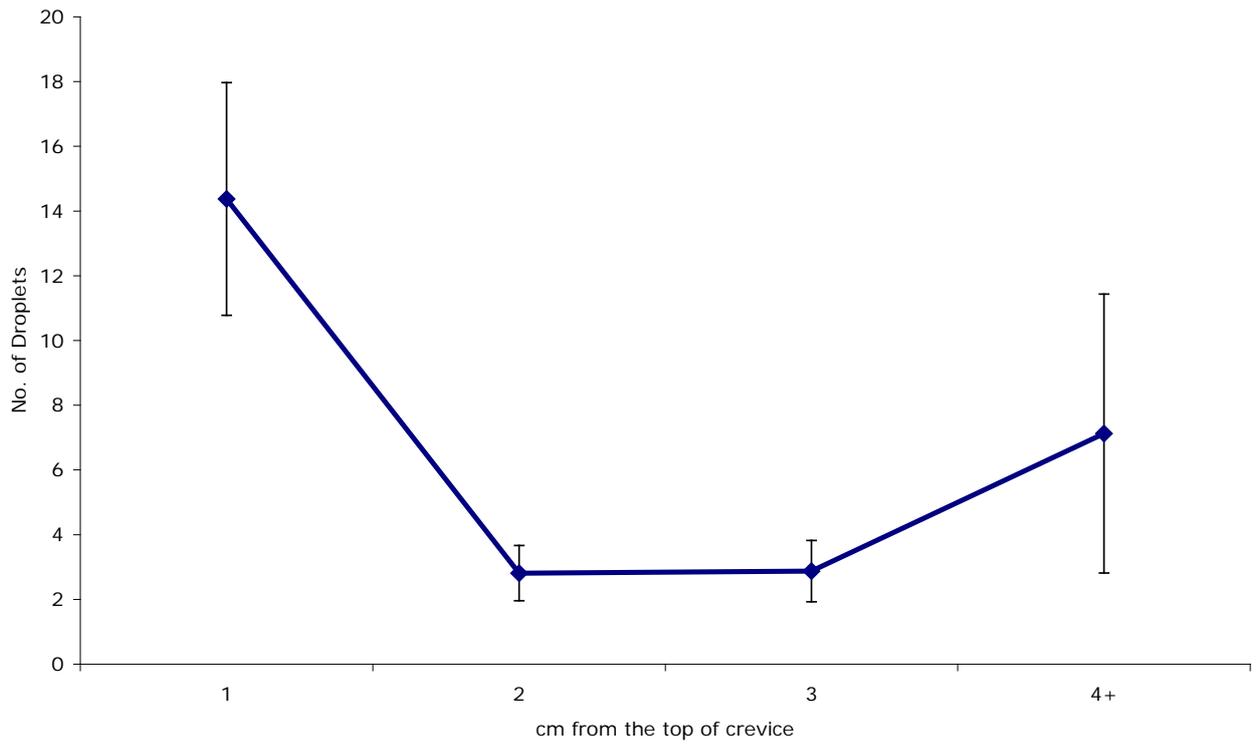


Figure 52. Average number of droplets penetrating the crevices in the oil treatment. Results are given in cm from the top of the crevice (± 1 SE).

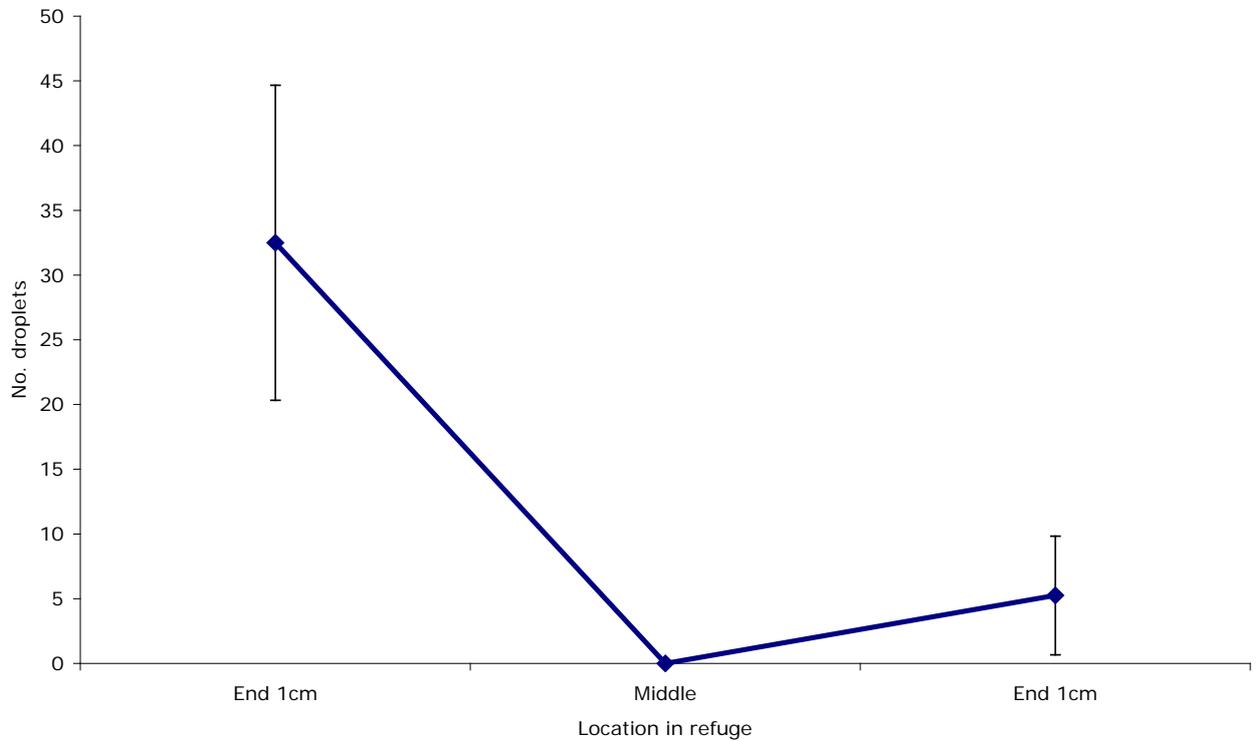


Figure 53. The number of droplets penetrating the refuge crevices held horizontally in the oil treatment.

Trial 2

The batch of isolate IMI 389521 produced by Somycel S.A. for the purpose of the trial showed a good level of viability (90.6% germination) and no contaminants were present. The final product contained 7.6×10^{10} conidia/g and had a moisture content of 8.36%.

The average temperature recorded in the grain store throughout the trial was 16.0°C, with a minimum of 6.7°C and a maximum of 24.4°C. There was very little variation in temperature observed between the ten arenas (Figure 54). The average humidity during the trial recorded using the Tinytag dataloggers was 77.1%, with a minimum of 45.2% and a maximum of 96.0% (Figure 55).

The exact quantity of the oil formulation delivered to each replicate could not be determined directly, but an indication was provided by measuring the volume of oil and the number of conidia delivered by the sprayers immediately after treatment of each replicate ring. This indicated that the quantity of the blank oil formulation delivered to each ring showed only a slight variation (mean \pm SE 2.6613 ± 0.0465 g) (Table 31). Some variation was also seen for the delivery of the conidia to the target areas. For the lower concentration ($2.5 \times 10^{10}/\text{m}^2$), the amount of conidia delivered compared fairly well with the number required (Table 31). However, for the higher concentration ($5.1 \times 10^{10}/\text{m}^2$) the amount of conidia was higher than expected for all replicates (Table 31).

The viability of the conidia in both the oil and dust formulations remained very high throughout the duration of the trial, with greater than 80% germination observed for both formulations for conidia recovered from the treated plywood squares (Table 32).

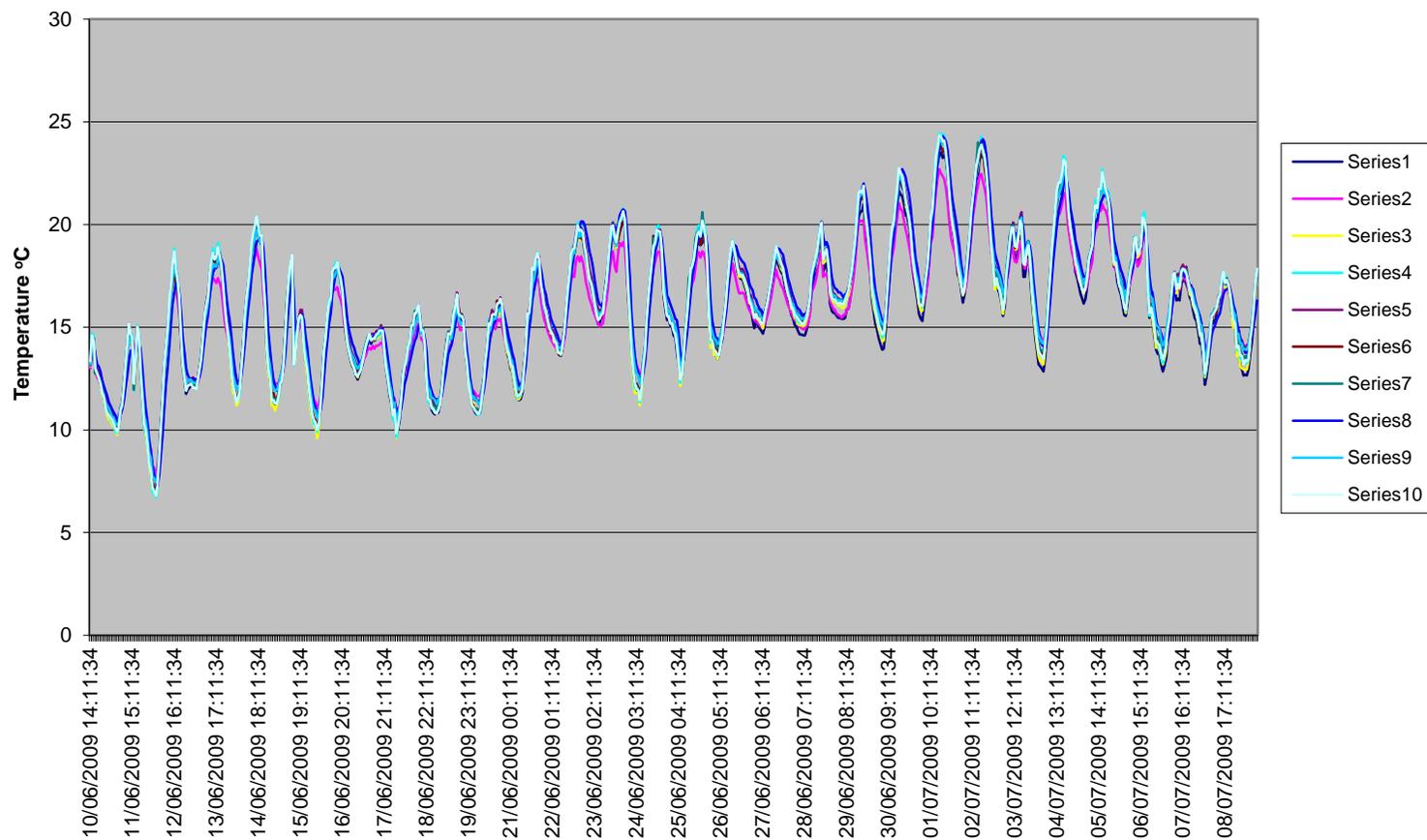


Figure 54. Temperatures recorded using the Squirrel datalogger in each of the ten arenas throughout Trial 2.

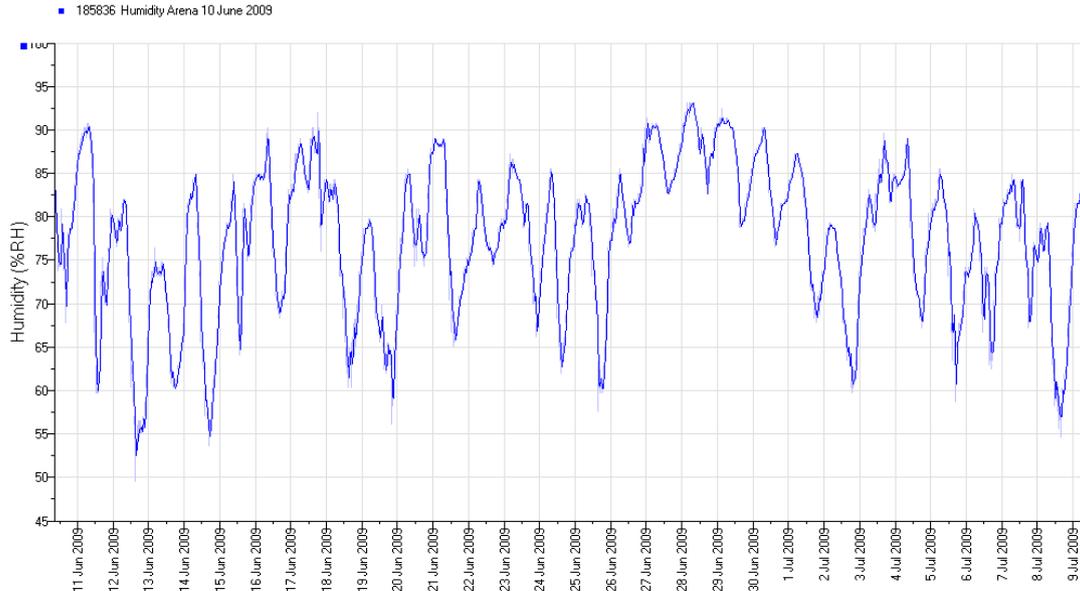


Figure 55. An example trace showing the humidity recorded within a plywood arena during Trial 2 from treatment to final assessment.

Table 31. Amount of oil formulation delivered to individual replicates within arenas. For the control oil formulation the figure shows the weight of oil (g) delivered. For the oil formulations containing conidia the figure shows the number of conidia/m².

Arena Number	Treatment	Weight applied
2	Blank oil	2.5839g
3	Blank oil	2.5351g
4	Blank oil	2.6647g
6	Blank oil	2.7870g
9	Blank oil	2.7357g

Arena Number	Target concentration	Conidia applied
1	$2.5 \times 10^{10}/m^2$	$2.0 \times 10^{10}/m^2$
4	$2.5 \times 10^{10}/m^2$	$2.4 \times 10^{10}/m^2$
5	$2.5 \times 10^{10}/m^2$	$2.3 \times 10^{10}/m^2$
6	$2.5 \times 10^{10}/m^2$	$3.1 \times 10^{10}/m^2$
8	$2.5 \times 10^{10}/m^2$	$3.0 \times 10^{10}/m^2$
2	$5.1 \times 10^{10}/m^2$	$1.0 \times 10^{11}/m^2$
3	$5.1 \times 10^{10}/m^2$	$9.7 \times 10^{10}/m^2$
7	$5.1 \times 10^{10}/m^2$	$8.1 \times 10^{10}/m^2$
8	$5.1 \times 10^{10}/m^2$	$7.1 \times 10^{10}/m^2$
9	$5.1 \times 10^{10}/m^2$	$7.6 \times 10^{10}/m^2$

Table 32. % germination of conidia recovered from plywood squares for the oil and dust formulations at three time points post-treatment.

Formulation and Arena Number	Day 1	Day 15	Day 29
Oil 3	90.4	87.6	84.9
Oil 7	85.7	88.2	85.2
Oil 9	86.9	86.1	87.8
Dust 5	85.4	83.3	83.2
Dust 4	86.6	86.3	84.2
Dust 8	85.3	81.5	82.7

There was a highly significant effect of treatment with the biopesticide formulations on mortality for all three species after 14 days exposure (GLM, $F_{6,28}=26.65$, $P<0.001$, $F_{6,28}=18.57$, $P<0.001$ and $F_{6,28}=28.25$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively) (Table 33 (A)). Subsequent analysis showed that at the 5% probability level there were no significant differences between the mortality recorded in the rings with no treatment and those that had been treated with either the oil or dust carriers ($P>0.05$) (Table 33 (A)). Mortality of *S. granarius* and *C. ferrugineus* with both the oil and the dust formulations containing conidia at either concentration caused significantly greater mortality in comparison to the untreated and oil and dust carrier treated rings ($P<0.05$) (Table 33 (A)). Mortality of *O. surinamensis* for the oil formulation containing 2.5×10^{10} conidia/m² was not significantly different from blank oil formulation or the no treatment control. However, mortality for the oil formulation containing 5.1×10^{10} conidia/m² and for both concentrations of the dust formulation was significantly higher than that for the no treatment or carrier controls (Table 33 (A)). Treatment with the dust formulation resulted in significantly greater mortality for *S. granarius* and *O. surinamensis* compared with treatment with the oil formulation at either concentration. When allowance was made for multiple comparisons (Bonferroni adjustment, $P= 0.0024$), there was no longer a significant difference between the dust and oil formulations containing the conidia for *S. granarius* and *C. ferrugineus*. *Oryzaephilus surinamensis* showed no significant difference in mortality resulting from treatment with the oil formulation at either concentration compared with the no treatment or blank formulation treatments when the Bonferroni adjustment was applied.

Treatment with pirimiphos methyl resulted in 100% mortality for all three species in all replicate treatments after 14 days. This was significantly different to the mortality for the three species on the water (control) treatment (Table 33 (B)).

Table 33. Derived mean % mortality of insects 14 days after exposure to different treatments. % mortality is expressed in terms of the number of insects recovered for each species. Figures in parentheses are the derived 95% confidence intervals. (A) Comparison of treatments and controls for the biopesticide

formulations. Oil blank is the oil carrier without conidia. Dust blank is Entostat alone. (B) Comparison of Actellic (pirimiphos methyl) treatment and control.

(A)

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	0.8 a (0.06, 10.1)	4.2 a,b (0.98, 16.5)	4.1 a (1.2, 13.1)
Oil blank	2.2 a (0.4, 10.6)	6.7 a,b (2.1, 19.2)	11.7 a (5.4, 23.5)
Dust blank	3.7 a (1.1, 11.7)	3.3 b (0.65, 15.5)	12.3 a (5.8, 24.2)
Oil 2.5 x 10 ¹⁰ /m ²	29.9 b (20.5, 41.3)	18.9 a, c (9.7, 33.6)	50.7 b (37.8, 63.5)
Oil 5.1 x 10 ¹⁰ /m ²	31.9 b (22.1, 43.4)	31.2 c (19.3, 46.4)	60.2 b,c (45.6, 73.2)
Dust 2.5 x 10 ¹⁰ /m ²	53.1 c (41.5, 64.3)	59.1 d (43.9, 72.8)	74.7 c (62.0, 84.2)
Dust 5.1 x 10 ¹⁰ /m ²	57.4 c (45.8, 68.1)	72.4 d (57.1, 83.8)	79.1 c (65.9, 88.2)

In each column, means followed by the same letter are not significantly different (GLM, P>0.05)

(B)

	Mean % mortality		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Water (control)	0.08 a (0.25, 1.14)	2.1 a (0.1, 10.1)	0.08 a (0.3, 1.2)
Actellic	100 b	100 b	100 b

In each column, means followed by the same letter are not significantly different (two sample t test, P>0.05)

There was a highly significant effect of treatment with the biopesticide formulations on mortality for all three species after 28 days exposure (GLM, $F_{6,28}=60.31$, $P<0.001$, $F_{6,28}=20.25$, $P<0.001$ and $F_{6,28}=35.47$, $P<0.001$ for *S. granarius*, *O. surinamensis* and *C. ferrugineus* respectively) (Table 34 (A)). Subsequent analysis showed that, at the 5% probability level, there were no significant differences between the mortality recorded in the rings with no treatment and those that had been treated with either the oil or dust carriers for *S. granarius* and *O. surinamensis* ($P>0.05$) (Table 34 (A)). Mortality of *C. ferrugineus* in the blank oil formulation treatment was significantly higher than for the rings receiving no treatment. Both the oil and the dust formulations, at either concentration, caused significantly higher mortality of all three species in comparison to the untreated and oil and dust carrier treated rings ($P<0.05$) (Table 34 (A)). When allowance was made for multiple

comparisons (Bonferroni adjustment $P= 0.0024$), there was no longer a significant difference between the dust and oil formulations for any of the three species tested.

Only a few additional insects were recovered from the rings treated with pirimiphos methyl at the 28-day assessment, all of which were dead (Table 34 (B)). There were a few additional dead insects for each species for the water treatments (Table 34 (B)).

After each assessment, the dead insects recovered from the rings and the refuges were surface sterilized and held on a damp filter paper in a Petri dish. Subsequent examination showed evidence of death by mycosis for the majority of insects exposed to the treatments with IMI 389521 (Figure 56). There was no evidence of mycosis for insects from the control, carrier formulations or the pirimiphos methyl treatments (Figure 56).

Table 34. Derived mean % mortality of insects 28 days after exposure to different treatments. % mortality is expressed in terms of the cumulative number of dead insects recovered after 14 and 28 days divided by the total of the number of insects recovered after 28 days and the number of dead insects after 14 days. Figures in parentheses are the derived 95% confidence intervals. (A) Comparison of treatments and controls for the biopesticide formulations. Oil blank is the oil carrier without conidia. Dust blank is Entostat alone. (B) Comparison of Actellic (pirimiphos methyl) treatment and control.

(A)

	Mean % mortality*		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Control (no treatment)	1.7 a (0.3, 9.5)	11.4 a (4.5, 26.1)	8.7 a (3.5, 20.2)
Oil blank	4.7 a (1.6, 13.2)	12.4 a (5.3, 26.4)	26.0 b (15.7, 39.9)
Dust blank	5.8 a (2.2, 14.2)	9.3 a (3.4, 22.8)	21.5 a, b (12.1, 35.3)
Oil $2.5 \times 10^{10}/m^2$	53.6 b (42.2, 64.6)	45.3 b (30.5, 61.0)	79.2 c (65.9, 88.2)
Oil $5.1 \times 10^{10}/m^2$	56.5 b (44.9, 67.3)	52.5 b (37.4, 67.2)	79.8 c (66.1, 88.9)
Dust $2.5 \times 10^{10}/m^2$	90.6c (81.4, 95.5)	83.1 c (68.4, 91.8)	94.1 d (83.5, 98.1)
Dust $5.1 \times 10^{10}/m^2$	90.9 c (82.1, 95.6)	79.8 c (64.8, 89.4)	95.1 d (84.6, 98.6)

In each column, means followed by the same letter are not significantly different (GLM, $P>0.05$)

(B)

	Mean % mortality*		
	<i>S. granarius</i>	<i>O. surinamensis</i>	<i>C. ferrugineus</i>
Water (control)	1.6 a (0.1, 4.7)	5.1 a (0.3, 15.4)	1.8 a (0.9, 13.0)
Actellic	100 b	100 b	100 b

In each column, means followed by the same letter are not significantly different (two sample t test, $P > 0.05$)

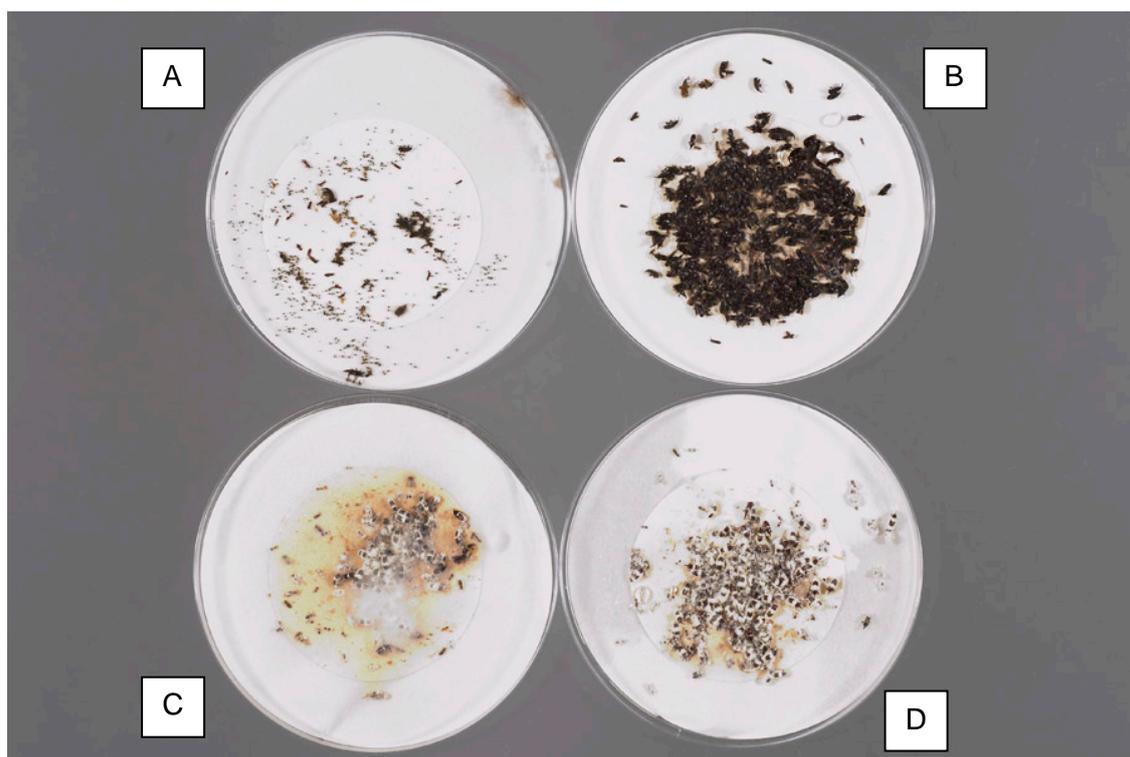


Figure 56. Evidence of mortality as a result of mycosis. Dead insects were removed from the steel rings, surface sterilized and placed on damp filter paper in a Petri dish. Fungal growth was observed on insects from IMI 389521 treatments only. A. Insects from rings with no treatment (control). B. Insects from rings treated with pirimiphos methyl C. Insects from rings treated with conidia in oil formulation D. Insects from rings treated with conidia in dust formulation.

Discussion

Evidence that the fungal formulations have a good level of efficacy under practical conditions is essential if biopesticides based on *B. bassiana* are to be considered for taking forward for product registration in the future. The pilot scale trials were carried out at the time of year when it may be expected that farmers are preparing the stores for incoming grain from harvest and, therefore, when stores are likely to be empty. Monitoring of the environmental conditions showed that for both trials, the average temperature of approx. 15.5°C was lower than the temperature used for most of the laboratory bioassays (20°C). The average relative humidity was higher in Trial 1 than for Trial

2, but the average humidity in both trials was higher than that for the laboratory bioassays (70%). These environmental conditions are representative of those likely to be found in UK farm grain stores pre-harvest and it would be necessary for the fungus to provide good levels of control under these conditions. The pilot scale trial also enabled practical considerations of the application of the formulations to be assessed and measurement of likely contamination of untreated areas to be made.

The importance of monitoring spray deposition was apparent in the first pilot scale trial. By monitoring deposition of the conidia in the oil formulation, we found that only 1.1×10^8 of viable conidia were deposited per m^2 , indicating that the spray technique needed improvement. Possible problems may have been due to the nozzle blocking during application (although preliminary trials had shown this not to be the case) or potentially that the walking speed at application should be reduced. The size of the test arenas may also have prevented effective deposition. Comparing the different methodologies to assess deposition (selective agar plates and Shellsol 'traps'), it can be seen that there is no significant difference between taking readings from the haemocytometer (Shellsol 'traps') or the CFU counts. The CFU counts are 94% of the haemocytometer counts. The slight difference in the readings can be accounted for by the viability of the conidia. Viability at the time of the trials was assessed as 91.1%, which accounts for the difference. Overall, the oil sensitive paper and lumogen tracers showed that there was a good even distribution of droplets over the target area and that the applied droplets were capable of penetrating cracks and crevices. The Shellsol traps also showed that the level of airborne inoculum landing in the traps reduced back to below the level of background contaminants after 2 days, showing that the spray is adhering to the surfaces to which it was applied.

The amount of the dust formulation applied to the steel rings and to the bait stations was also assessed. It was found that the amount of conidia applied to the plywood arenas in the dust formulation (1.46×10^{11} conidia/ m^2) was much greater than that applied in the oil formulation (1.1×10^8 conidia/ m^2).

In Trial 1, it was found that the dust formulation, either applied directly to the plywood arena or in a bait station, resulted in significantly higher mortality of all three storage beetle species after 21 days compared with the oil formulation. However, it should be borne in mind that the different application methods resulted in differences in the amount of conidia to which the insects were exposed in each treatment. Therefore, the greater mortality caused by the dust formulation may have been as a result of the greater concentration of conidia. It was also seen that at each assessment point the numbers of dead insects of all three species was very similar when the insects that were treated directly and the insects introduced 24 h after treatment were compared. This shows that it is possible for the insects to pick up the conidia from a treated surface in

sufficient numbers to produce mortality. This applied to both formulations and represents a significant improvement to the findings from project LK0914, where only insects that were sprayed directly eventually died (Cox *et al.*, 2004).

Oryzaephilus surinamensis and *C. ferrugineus* that were removed from the oil formulation treatments two hours after treatment showed significant levels of mortality after 14 days at 20°C, 70% r.h. Mortality for these insects after 14 days was higher than that for insects that remained in the plywood arenas. This indicates that the lower temperature in the store may have affected insect mortality, perhaps by reducing the speed of kill of the fungus.

The dust formulation showed a good level of viability on the three different surface types over the 42-day period both in terms of the germination of the conidia and the level of insect mortality produced. Interestingly, although the dust formulation on the concrete showed the greatest fall in the % germination at day 9, mortality was higher for insects on the concrete compared to the wood and metal. The oil formulation also maintained a good level of viability, although due to the fewer conidia present, this was more difficult to demonstrate particularly with regard to insect mortality. The dust formulation in the bait stations continued to cause high levels of insect mortality throughout the experiment. It will be noted that at each assessment point, the PC trap resulted in higher levels of mortality than the Exosect trap. This was due to the trap styles and the ease of entry and exit for the insects. It was found that the majority of the insects were present in the PC trap at the 14-day assessment point, whereas insects were found both in the arena and in the Exosect trap. It is likely that even though the PC trap did not have the usual Fluon coating to prevent insect escape, insects found it difficult to leave the trap once they had entered. It is therefore likely that these insects were exposed to greater concentrations of the conidia compared to insects visiting the Exosect trap and this would explain the higher levels of mortality observed.

The second trial was designed to determine the effectiveness of the two different formulations containing isolate IMI 389521 at the same concentrations. In addition, a currently registered chemical pesticide was included as a positive control. The chemical pesticide, Actellic (pirimiphos methyl) when applied at the recommended concentration caused rapid death of all three species of insect. Large numbers of knock-down or dead insects were observed within two hours of the introduction of the insects to the treated surface and 100% mortality was recorded for insects recovered from the rings after 14 days.

The biopesticide formulations also caused a significantly greater level of mortality than was observed for the control treatments. In general, the dust formulations gave a greater level of control than the oil formulation. This may have been due primarily to the formulation, but may also be a result of the method of application. As only a small area could be treated using the experimental

design described, it was not possible to use conventional application equipment for delivery of the formulations due to the very small volumes/weights that needed to be applied. A hand-held sprayer was, therefore, used for application of the oil formulation. This had the disadvantage that coverage across the surface was not uniform and some small areas may have remained untreated. A larger scale trial should consider comparison of the two formulations with the oil formulation applied using appropriate spray equipment to provide a uniform coverage. It should be borne in mind that application of the oil formulation at the higher concentration is likely to have resulted in a greater concentration of conidia than the expected 5.1×10^{10} conidia/m². This would have been higher than the equivalent concentration of the dust formulation, and may suggest that the oil formulation is not as effective as the dust when applied to plywood.

After 28 days exposure to the dust formulations, the mortality for all three species was similar. Exposure to the oil formulations resulted in greater mortality of *C. ferrugineus* than for the other two species. The viability of the conidia as determined by the % germination remained high throughout the trial indicating that under the test conditions isolate IMI 389521 retained the potential to infect insects and may, therefore, have residual activity. Evidence for insect mortality as a result of mycosis was observed for dead insects recovered from rings treated with the formulations containing IMI 389521, confirming that mortality was caused by the entomopathogenic fungus.

Comparison of the two concentrations for both the oil and the dust formulations showed little difference in the level of mortality recorded for all three species. It is, therefore, possible that a lower, more economically viable, concentration would result in a similar level of mortality. This should be investigated further in a larger scale trial, which would also allow a more appropriate testing of the oil formulation, which cannot be carried out well in such small arenas.

In conclusion, we have shown that:

- Application of *B. bassiana* IMI 389521 under typical UK grain storage conditions results in significant levels of mortality for *S. granarius*, *O. surinamensis* and *C. ferrugineus*.
- The dust formulation would appear to be more effective than the oil formulation.
- Both formulations retain a good level of viability under typical UK grain store conditions for up to 42 days.
- Oil formulations are able to penetrate into cracks and crevices
- Bait stations could be used to contain dust formulations
- Larger scale trials are needed to further explore the differences between the dust and oil formulations and to ensure that control can be achieved at concentrations that are economically viable.

3.5.4. Step 4.4: Versatility and use of the biopesticide

Introduction

A novel insect control agent for use in UK grain stores should, ideally, integrate with current procedures for best practice. Therefore, the control agent should form part of an integrated pest management (IPM) approach. The cost effectiveness of any novel agent is a key factor in determining likely future commercial exploitation. The development of the mass production methods within this project and determination of the likely maximum quantity for use of the agent on its own have provided data that can be used to examine the likely costs to produce the agent. The cost of the use of the agent on its own should also be considered with the costs of use in combination with another control method. This may also increase the versatility of use of the biological control agent. It is known that the use of a diatomaceous earth (DE) structural treatment has potential against the saw-toothed grain beetle, *O. surinamensis* (Cook *et al.*, 2003; 2004) and it has been reported that a commercial DE synergises the effect of unformulated conidia of *B. bassiana* against some storage pests (Lord, 2001; Akbar *et al.*, 2004). The effects of the biopesticide formulation and DE when used alone at a single concentration were compared to the effect of the combination by exposing *O. surinamensis* in treated Petri dishes to provide information on the versatility of the biopesticide for potential users.

Materials and methods

A preliminary experiment was undertaken to determine appropriate concentrations of both the DE and *B. bassiana* to use that would show any additive or synergistic effects on the mortality of *O. surinamensis*. Silico-sec was chosen as the representative DE and *B. bassiana* IMI 389521 was used. Glass Petri dishes (90 mm diameter) were treated with Silico-sec at a concentration of 0.1, 1 or 10 g/m². *Beauveria bassiana* IMI 389521 was applied at 1x 10¹⁰ conidia/m² or 1 x 10¹¹ conidia/m². Adult *O. surinamensis* (Tram) (20) were placed in each Petri dish for a period of 24 h at 20°C, 70% r.h. There were five replicates for each treatment and concentration. After 24 h, the insects were transferred to clean Petri dishes with a small quantity of food. Mortality was assessed after 14 days.

Following the preliminary experiment, the effect of a mixture of the DE and IMI 389521 was assessed at two temperature and humidity combinations. The concentrations selected for the treatments were 0.1 g/m² of Silico-sec and 1 x10¹⁰ conidia/m² of IMI 389521. The treatments (Silico-sec alone, IMI 389521 alone or a mixture of Silico-sec and IMI 389521) were applied to glass Petri dishes and twenty adult *O. surinamensis* were introduced to each dish. There were five replicate dishes for each treatment. The efficacy was assessed at 15°C, 80% r.h., representing typical conditions in a UK grain store pre-harvest and a 20°C, 70% r.h., representing the conditions of previous laboratory bioassays. After 24 h, insects were transferred to clean Petri dishes with a small amount of food. Mortality was assessed 14 days after treatment.

Expected mortality for the combination of the DE and IMI 389521 was calculated with the formula $P_e = P_1 + P_2(1 - P_1)$ where P_e = expected mortality, P_1 = mortality from *B. bassiana* and P_2 = mortality from DE. Significant synergism was detected using a chi-square test (1 df, $\alpha = 0.05$, X^2 value > 3.84).

Results

A preliminary experiment was undertaken to determine the most appropriate concentration of the DE and the fungus to be used. A concentration dependant affect was seen for both the DE and IMI 389521. Treatment with IMI 389521 for 24 h at a concentration of 1×10^{11} conidia/m² resulted in < 90% mortality of *O. surinamensis* (Figure 57). Similarly, high levels of mortality were found in the Silico-sec treatments at 1 g/m² and 10 g/m² (Figure 57).

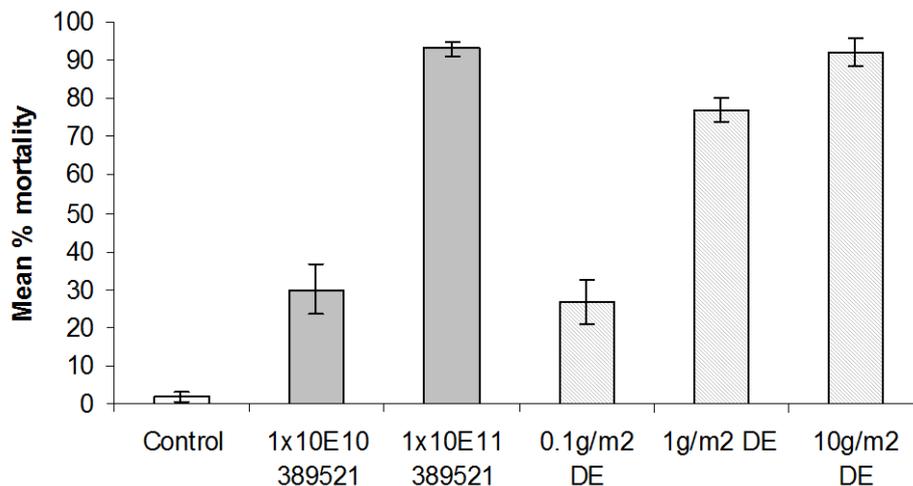


Figure 57. Mean (\pm SE) % mortality of *O. surinamensis* 14 days after exposure to different concentrations of the diatomaceous earth, Silico-sec (hatched bars), or *B. bassiana* IMI 389521 (solid bars) for 24 hours.

A mixture of Silico-sec and IMI 389521 resulted in higher levels of mortality than treatment with the fungus or the DE alone at both temperatures and significant synergism was indicated (X^2 value = 458.2 and 89.48 at 15°C and 20°C, respectively, $P < 0.05$) (Figure 58). Mortality of *O. surinamensis* treated with Silico-sec was very similar at both temperatures. Treatment with IMI 389521 resulted in significantly greater mortality at 20°C, 70% r.h. than was observed at 15°C, 80% r.h. Treatment with the combination of the DE and IMI 389521 also resulted in significantly greater mortality at 20°C, 70% r.h. compared with that at 15°C, 80% r.h. ($P < 0.01$) (Figure 58).

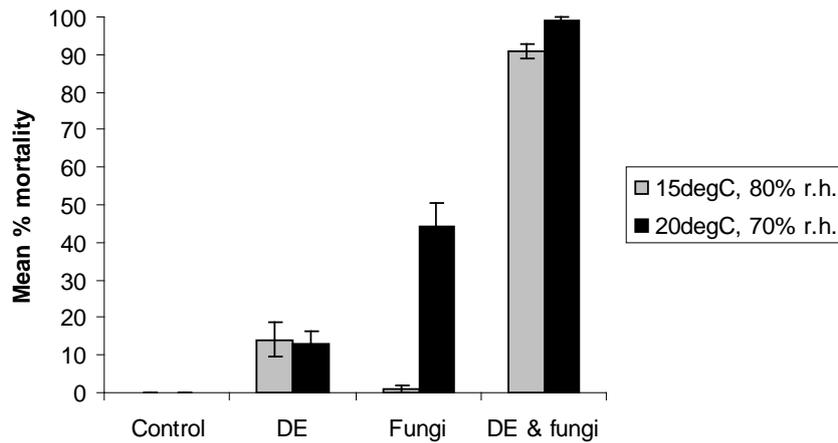


Figure 58. Mean (\pm SE) % mortality of *O. surinamensis* 14 days after exposure to the diatomaceous earth, Silico-sec, *B. bassiana* IMI 389521 or a combination of the DE and IMI 389521 for 24 hours.

Discussion

The preliminary experiment determined appropriate concentrations of the DE and the fungus to be used. It was important that, although mortality was observed, this should be low in order that any additive or synergistic effects of the fungus and the DE could be seen. The recommended application rate of a DE for a structural treatment is 10 g/m². This resulted in very high levels of mortality of *O. surinamensis*, approaching 100%. A concentration of 1 g/m² Silico-sec also resulted in high mortality levels, but a concentration of 0.1 g/m² Silico-sec resulted in approximately 27% mortality, which would enable any enhancements achieved with the addition of the fungus to be observed. Similarly, the level of mortality resulting from the treatment with 1 x 10¹⁰ conidia/m² (approximately 30%) also enabled possible enhancements in efficacy to be determined.

It has been found that diatomaceous earths are less effective at high humidities, whilst it is known that entomopathogenic fungi require a high humidity. By combining the two, the potential exists to optimise the range of environmental conditions at which the products are effective (Akbar *et al.*, 2004). In the current study, the greater efficacy of the fungus alone at 70% r.h. compared with 80% r.h. is likely to be due to the different temperatures used, as the fungus will be more effective at 20°C compared with 15°C. No difference was seen in the effectiveness of the DE at the temperature and humidities used and this may be due to the narrow range studied.

The combination of IMI 389521 and Silico-sec resulted in a significant increase in the overall mortality in comparison with either treatment alone at both 15°C and 20°C. At both temperatures, a significant synergism was indicated and this was particularly apparent at 15°C. A synergistic effect of a diatomaceous earth mixed with *B. bassiana* has been reported previously (Lord, 2001; Akbar *et al.*, 2004).

Previous studies that have examined the combination of an entomopathogenic fungus and a DE have examined the effect when applied directly to grain (Lord, 2001; Akbar *et al.*, 2004; Lord, 2005; Michalaki *et al.*, 2006; Athanassiou and Steenberg, 2007, 2008). The potential effect of this combination as a structural treatment has not previously been examined. Many of the studies cited above have also examined the effect at high temperatures (25 to 34°C) that are unlikely to be encountered in the UK. The current study has therefore shown that a combination of a diatomaceous earth and IMI 389521 has potential as a structural treatment under conditions likely to be found in a UK grain store.

3.6. Overall conclusions

During the course of the project, significant progress was made, leading to the fulfilment of all four main objectives. Of particular note is that enhancement of the production and formulation of the conidia has negated a need for a period where the humidity needs to be close to 100% and the conidia do not have to be directly applied to the insects to achieve good efficacy. In addition, mass production methods resulting in consistent, high quality production of conidia with excellent viability and virulence have been determined and significant control of insect populations under practical conditions has been demonstrated.

This research has made a significant step towards the development of a biopesticide, based on *B. bassiana*, as a structural treatment in UK grain stores. In addition to overcoming the main technical obstacles, information has also been collected on the concerns and issues that the development and use of a biopesticide may generate. The research undertaken within this project has, as far as was possible, addressed these concerns and generated important data to demonstrate that this type of product can be used in a practical situation to achieve good levels of control. Information has also been gathered on the registration process for a biopesticides. This will be important in the future development of the product and provides a clear foundation to determine the further studies that would be necessary.

The project has demonstrated that a biopesticide based on *B. bassiana* has potential for control of stored product insects in UK grain stores. Candidate formulations have been identified but further work will be needed to fully establish the most appropriate formulation. The mass production process has been optimised, but until the most appropriate formulation and dose rate have been established, it will remain to be seen whether cost effective production can be realised. The project has made significant progress in the development of a novel structural treatment that would be a benefit to UK farmers and, in particular, those in the organic sector.

3.6.1. Recommendations for further work

This project has demonstrated, at a small scale, the potential of a biopesticide based on *Beauveria bassiana* as a structural treatment for UK grain stores to control a range of beetle pest species.

Recommendations for further studies that would improve our understanding of this potential or that may lead to the development of a full registration package are shown below:

Further studies on the formulation are needed and, in particular, consideration of the application of a dust formulation, formulation development (particularly further testing of efficacious and economically viable concentrations and ratios of carrier to spores) and the potential of bait stations should be considered.

Large-scale trials are required for product registration. The design of these trials should be developed in consultation with CRD to ensure that it would fulfil requirements for any future registration package.

Examination of effects on population development and other target pests should be considered.

The potential for use as a treatment applied to grain should be considered.

Other studies, such as product toxicity, which may be required for a registration package.

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3.8. References

- Adane, K., Moore, D. and Archer, S. A. (1996). Preliminary studies on the use of *Beauveria bassiana* to control *Sitophilus zeamais* in the laboratory. *Journal of Stored Products Research*, 32: 105-113.
- Adler, C., Ojimekwe, P. and Tapondjou, A.L. (2000). Utilisation of phytochemicals against stored product insects. *Integrated Protection of Stored Products, IOBC Bulletin*, 23: 169-175.
- Aggarwal, K.K., Tripathi, A.K., Ahmed, A. Prajapati, V., Verma, N. and Kumar, S. (2001). Toxicity of L-Menthol and its derivatives against four storage insects. *Insect Science Applications*, 21: 229-235.
- Akbar, W., Lord, J.C, Nechols, J.R. and Howard, R.W. (2004). Diatomaceous earth increases the efficacy of *Beauveria bassiana* against *Tribolium castaneum* larvae and increased conidial attachment. *Journal of Economic Entomology*, 97: 273-280.
- Altre, J.A. and Vandenberg, J.D. (2001). Penetration of cuticle and proliferation in hemolymph by *Paecilomyces fumosoroseus* isolates that differ in virulence against lepidopteran larvae. *Journal of Invertebrate Pathology*, 78: 81-86.
- Alves, L.F., Oliveira, D.G. and Neves, P.M. (2008). Factors affecting diatomaceous earth effectiveness in the control of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) adults. *Neotropical Entomology*, 37: 716-722.
- Anon. (2002). *Occupation Exposure Limits. EH40/2002*. Health and Safety Executive, Sudbury, Suffolk, UK., 106 pp.
- Armsworth, C.G., Baxter, I.H., Barton, L.E.E., Poppy, G.M. and Nansen, C. (2006). Effects of adhesive powders on the mating and flight behavior of Mediterranean fruit fly (Diptera: Tephritidae). *Journal of Economic Entomology*, 99: 1194–1202.
- Armsworth, C.G., Rogers, C.D., Barton, L.E.E., Soares, C. and Poppy, G.M. (2008). Uptake of adhesive powders from lure stations by Mediterranean fruit fly (Dipt., Tephritidae). *Journal of Applied Entomology*, 132: 45-53.
- Athanassiou, C.G. (2004). Feasibility of using *Beauveria bassiana* plus diatomaceous earth against three stored-product beetle species. In: L. Stengard-Hansen, M. Wakefield, J. Lukáš and V. Stejskal (Eds.). *Proceedings of the 4th meeting of COST Action 842 Working Group 4*, Athens, 2004. pp 50-52.
- Athanassiou, C.G. and Steenberg, T. (2007). Insecticidal effect of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) in combination with three diatomaceous earth formulations against *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). *Biological Control*, 40: 411-416.

- Athanassiou, C.G., Kavallieratos, N.G., Vayias, B.J., Tsakiri, J.B., Mikeli, N.H., Meletsis, C.M. and Tomanovic, Z. (2008). Persistence and efficacy of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) and diatomaceous earth against *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) on wheat and maize. *Crop Protection*, 27: 1303-1311.
- Austwick, P.K.C. (1980). The pathogenic aspects of the use of fungi; the need for risk analysis and registration of fungi. In: B. Lundholm and M. Stackerud (Eds). *Environmental Protection and Biological Forms of Control of Pest Organisms*. Ecological Bulletin (Stockholm) no. 31, p. 91-102.
- Barson, G., Fleming, D.A. and Allan, E. (1992). Laboratory assessment of the behavioural responses of residual populations of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) to the contact insecticide pirimiphos-methyl by linear logistic modelling. *Journal of Stored Products Research*, 28: 161-170.
- Barton, L.E.E., Armsworth, C.G., Baxter, I.H., Poppy G.M., Gaunt, L.F. and Nansen, C. (2006). Adhesive powder uptake and transfer by mediterranean fruit flies, *Ceratitidis capitata* (Diptera: Tephritidae). *Journal of Applied Entomology*, 130: 257-262.
- Bateman, R. and Chapple, A. (2001). The spray application of mycopesticide formulations. In: T. M. Butt, C. Jackson and N. Magan (Eds). *Fungi as Biocontrol Agents*. CABI International, Wallingford, UK, p 289-309.
- Bateman, R., Carey, M., Moore, D. and Prior, C. (1993). The enhanced infectivity of *Metarhizium flavoviride* in oil formulations to desert locusts at low humidities. *Annals of Applied Biology*, 122: 145-152.
- Baxter, I.H. (2008). Entomopathogen based autodissemination for the control of *Plodia interpunctella* (Hübner) – an examination of the critical components. PhD Thesis University of Southampton.
- Baxter, I.H., Howard, N., Armsworth, C.G., Barton, L. E. E. and Jackson, C. (2008). The potential of two electrostatic powders as the basis for an autodissemination control method of *Plodia interpunctella* (Hübner). *Journal of Stored Products Research*, 44: 152-161.
- Berry, I.L. (1973). Improved system for measuring flying activity of insects by detecting static charges. *Journal of Economic Entomology*, 66: 820-822.
- Bidochka, M.J. (2001). Monitoring the fate of biocontrol fungi. In: T. M. Butt, C. Jackson and N. Magan (Eds). *Fungi as Biocontrol Agents*, CAB International, Wallingford, UK, p. 193-218.
- Boucias, D.G., Pendland, J.C. and Latge, J.P. (1988). Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied and Environmental Microbiology*, 54: 1795-1805.
- Boyetchko, S., Pederson, E., Punja, Z. and Reddy, M. (1999). Formulations of Biopesticides. In: F.R. Hall and J.J. Menn (Eds). *Methods in Biotechnology*, Totowa, NJ, Humana Press. vol 5: *Biopesticides: Use and Delivery*: p 487-508.

- Brower, J.H., Smith, L., Vail, P.V. and Flinn, P.W. (1996). Biological control. In: B.H. Subramanyam and D.W. Hagstrum (Eds.). Integrated management of insects in stored products, Marcel Dekker, New York, pp. 223-286.
- Butt, T.M., Ibrahim, L., Clark, S.J. and Beckett, A. (1995). The germination behaviour of *Metarhizium anisopliae* on the surface of aphid and flea beetle cuticles. *Mycology Research*, 99: 45-950.
- Bridgeman, B. (1994). Structural treatment with amorphous silica slurry: an integral component of GRAINCO's IPM strategy. In: E. Highley, E.J. Wright, H.J. Banks and B.R. Champ (Eds). Proceedings of the 6th International Working Conference on Stored-product Protection, Canberra, Australia, CAB International, Wallingford, UK., pp. 628-630.
- Bridgeman, B. (2000). Application technology and usage patterns of diatomaceous earth in stored product protection. In: Zuxun, J., Quan, L., Yongsheng, L., Xianchang, T., Lianghua, G., (Eds). Proceedings of the 7th International Working Conference on Stored-product Protection, Beijing, China, Sichuan Publishing house of science and technology, Chengdu, Sichuan Province, Peoples Republic of China, pp. 785-789.
- Chander, H., Nagender, A., Ahuja, D. K. and Berry, S.K. (1999). Laboratory evaluation of plant extracts as repellents to the rust red flour beetle, *Tribolium castaneum* (Herbst), on jute fabric. *International Pest Control*, 41: 18-20.
- Chander, H., Nagender, A., Ahuja, D. K. and Berry, S.K. (2000). Repellency of different plant extracts and commercial formulations used as prophylactic sprays to protect bagged grain against *Tribolium castaneum* – a field study. *Journal of Food Science and Technology*, 37: 582-585.
- Charnley, A.K. and St. Leger, R.J. (1991). The role of cuticle-degrading enzymes in fungal pathogenesis of insects. In Cole, G and Hoch, H.C. (Eds). The fungal spore and disease initiation. Plenum Press, New York, pp. 267-286.
- Cherry, A.J., Abalo, P. and Hell, K. (2005). A laboratory assessment of the potential of different strains of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuilleum and *Metarhizium anisopliae* (Metschnikoff) to control *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in stored cowpea. *Journal of Stored Products Research*, 41: 295-309.
- Cherry, A.J., Aba, P., Hel, K. and Korie, S. (2007). Farm-scale trials to compare the entomopathogenic fungus *Beauveria bassiana* with pirimiphos methyl plus deltamethrin and essential oil of lemongrass for protection of stored cowpea against *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Annals of applied Biology*, 151: 1-10.
- Cline, L.D., Zettler, J.L., McDonald, L.L. and Highland, H.A. (1984). Continuous exposure to sublethal doses of synergised pyrethrins, effects on resistance and repellency in *Tribolium confusum* (Coleoptera: Tenebrionidae). *Journal of Economic Entomology*, 77: 1189-1193.

- Collins, L.E. and Chambers, J. (2003). The I-SPY Insect Indicator: an effective trap for the detection of insect pests in empty stores and on flat surfaces in the cereal and food trades. *Journal of Stored Products Research*, 39: 277-292.
- Collins, L.E., Bryning, G.P., Wakefield, M.E., Chambers, J. and Cox P.D. (2007). Progress towards a multi-species lure: Identification of components of food volatiles as attractants for three storage beetles. *Journal of Stored Product Research*, 43: 53-63.
- Collins, L.E., Bryning, G.P., Wakefield, M.E., Chambers, J. Fennah, K. and Cox, P.D. (2008). Effectiveness of a multi-species attractant in two different trap types under practical grain storage conditions. *Journal of Stored Product Research*, 44: 247-257.
- Collins, P.J., Lambkin, T.M., Bridgeman, B.W. and Pulvirenti, C. (1993). Resistance to grain-protectant insecticides in coleopterous pests of stored cereals in Queensland, Australia. *Journal of Economic Entomology*, 86: 239-245.
- Cook, D.A., Collins, L.E. and Armitage, D.M. (2003) Diatomaceous earth structural treatment against *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) under fluctuating UK conditions. *Proceedings of the 8th International Working Conference on Stored Product Protection*, 22-26 July, 2002 pp 675-679.
- Cook, D.A., Collins, D.A. and Collins, L.E. (2004). Efficacy of diatomaceous earths, applied as structural treatments, against stored product insects and mites. HGCA Project Report No. 344, 52 pp.
- Coombs, C.W. and Freeman, J.A. (1955). The insect fauna of an empty granary. *Bulletin of Entomological Research*, 46: 399-417.
- Copping, L.G. (Ed) (1998). *The BioPesticide Manual. A World Compendium*. Farnham, British Crop Protection Council (BCPC) pp. 333.
- Copping, L.G. (Ed.) (2004). *The Manual of Biocontrol Agents*. British Crop Protection Council, Bracknell, 752pp.
- Cox, P.D. (2004). Potential for using semiochemicals to protect stored products from insect infestation. *Journal of Stored Products Research*, 40: 1-25.
- Cox, P.D. and Wilkin, D.R. (1998). A review of the options for biological control against invertebrate pests of stored grain in the UK. *IOBC/WPRS Bulletin* 21 (3), 27-32.
- Cox, P.D, Parish, W.E. and Beirne, M.A. (1989). Variations in the refuge seeking behaviour of four strains of *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae) at different temperatures. *Journal of Stored Products Research*, 25: 239-242.
- Cox, P.D., Parish, W.E. and Ledson, M. (1990). Factors affecting the refuge-seeking behaviour of *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae). *Journal of Stored Products Research*, 26: 169-174.
- Cox, P.D., Atkinson, J.E., Bannon, K.L. and Hatfield, J. (1993). Studies of refuge-seeking behaviour and locomotory activity in *Cryptolestes ferrugineus* (Stephens) (Coleoptera:

- Cucujidae) using time-lapse video recordings. *Journal of Stored Products Research*, 29: 283-289.
- Cox, P.D., Clarke, P.G., Ford, H.L., Morgan, C.P. and Collins, L.E. (2000). Comparison of pesticide efficacy against insects and mites for grain store structure treatment. HGCA Project Report No. 233.
- Cox, P.D., Wakefield, M.E., Price, N., Wildey, K.B., Chambers, J., Moore, D., Aquino de Muro, M. and Bell, B.A. (2004). The potential use of insect-specific fungi to control grain storage pests in empty grain stores. HGCA Project Report No. 341, 49 pp.
- Dal Bello, G., Padin, S., Lopez Lastra, C. and Fabrizio, M. (2001). Laboratory evaluation of chemical-biological control of the rice weevil (*Sitophilus oryzae*) in stored grains. *Journal of Stored Products Research*, 37: 77-84.
- Dawson, A., Garthwaite, D.G. and Thomas, M.R. (2004a) Pesticide Usage Survey Report 192. Farm Grain Stores in Great Britain 2002/03. Defra: London 22pp.
- Dawson, A., Garthwaite, D.G., Anderson, H. and Thomas, M.R. (2004b) Pesticide Usage Survey Report 193. Commercial Grain Stores in Great Britain 2003. Defra: London 22 pp.
- De Faria, M.R. and Wraight, S.P. (2007). Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*, 43: 237-256.
- Desmarchelier, J.M. and Dines, J.C. (1987). Dryacide treatment of stored wheat: its efficacy against insects, and after processing. *Australian Journal of Experimental Agriculture*, 27: 309-312.
- Desmarchelier, J.M., Wright, E.J. and Allen, S.E. (1992). Dryacide : A structural treatment for stored-product insects. *Proceedings of the 5th Australian Applied Entomological Research Conference 1992*, 483-485.
- Doberski, J.W. (1981). Comparative laboratory studies on three fungal pathogens of the elm bark beetle, *Scolytus scolytus*: pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* to larvae and adults of *S. scolytus*. *Journal of Invertebrate Pathology*, 37: 188-194.
- Dytham, C. (2003). *Choosing and using statistics, a biologist's guide*. Oxford, UK, Blackwell Publishing. 248 pp.
- Ebeling, W. (1971). Sorptive dusts for pest control. *Annual Review of Entomology*, 16: 123-158.
- Egwunyenga, O. A., Alo, E.B. and Nmorsi, O.P.G. (1998). Laboratory evaluation of the repellency of *Dennettia tripetala* Baker (Annonaceae) to *Dermestes maculatus* (F.)(Coleoptera: Dermestidae). *Journal of Stored Products Research*, 34: 195-199.
- Fraenkel, G. and Blewett, M. (1943). The natural foods and the food requirements of several species of stored products insects. *Transactions of the Royal Entomological Society of London*, 93: 457-490.

- Glenn, D.M., Puterka, G.J., Vanderwet, T., Byers, R.E. and Feldhake, C. (1999). Hydrophobic particle films, a new paradigm for suppression of arthropod pests and plant diseases. *Journal of Economic Entomology*, 92: 759–771.
- Goettel, M.S. and Johnson, D.L. (1992). Environmental impact and safety of fungal biocontrol agents. In: C.J. Lomer and C. Prior (Eds). *Biological Control of Locusts and Grasshoppers, Proceedings of a workshop held at the International Institute of Tropical Agriculture, Cotonou, Republic of Benin, 29 April-1 May 1991*, CAB International, Wallingford, pp. 356-361.
- Goettel, M.S., Poprawski, T.J., Vandenberg, J.D., Li, Z. and Roberts, D.W. (1990). Safety to non-target invertebrates of fungal biocontrol agents. In: M. Laird, L.A. Lacey and E.W. Davidson (Eds). *Safety of Microbial Insecticides*, CRC Press, Boca Raton, pp 209-231.
- Haines, C.P. (1999). Arthropod natural enemies in stored products - overlooked and under-exploited. *Proceedings of the 7th International Working Conference on Stored Product Protection, Beijing, China, 1998*, 1205-1226.
- Hajek, A.E. and St. Leger, R.J. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, 39: 293-322.
- Haliday, W.R. and McGovern, T.P. (1987). Structure-activity relationships for repellency of alicyclic carboxamides to adult *Tribolium confusum*. *Journal of Agricultural Entomology*, 4: 289-296.
- Hallsworth, J.E. and Magan, N. (1994a). Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiology*, 140: 2705-2713.
- Hallsworth, J.E. and Magan, N. (1994b). Improved biological control by changing polyols/trehalose conidia of entomopathogens. *Brighton Crop Protection Conference-Pests and Diseases-1994*, Brighton: 1091-1096
- Hallsworth, J.E. and Magan, N. (1995). Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology*, 141:1109-1115.
- Hansen, L.S. and Steenberg, T. (2007). Combining larval parasitoids and an entomopathogenic fungus for biological control of *Sitophilus granarius* (Coleoptera: Curculionidae) in stored grain. *Biological Control*, 40: 237-242.
- Harper, S.H.T. and Lynch, J.M. (1981). Effects of fungi on barley seed germination. *Journal of General Microbiology*, 122: 55-60.
- Hidalgo, E., Moore, D and Le Patourel G. (1998). The effect of different formulations of *Beauveria bassiana* on *Sitophilus zeamais* in stored maize. *Journal of Stored Products Research*, 34: 171-179.
- Hong, T.D., Gunn, J., Ellis, R.H., Jenkins N.E. and Moore, D. (2001). The effect of storage environment on the longevity of conidia of *Beauveria bassiana*. *Mycological Research*, 105: 597-602.
- Hosnedl, V. and Honsová, H. (2002). Barley seed sensitivity to water stress at germination stage. *Rostlinná Výroba*, 48: 293-297.

- Hou, X., Fields, P.G. and Taylor, W. (2004). The effect of repellents on penetration into packaging by stored-product insects. *Journal of Stored Products Research*, 40: 47-54.
- Howse, P.E. and Underwood, K.L. (2000). Environmentally-safe pest control using novel bioelectrostatic techniques: initial results and prospects for area-wide usage, pp. 295-299. In K. H. Tan (ed.), *Area-wide control of fruit flies and other insects*. Penerbit University Sains Malaysia, Penang.
- Ignatowicz, S. and Wesolowska, B. (1994). Potential of common herbs as grain protectants: repellent effect of herb extracts on the granary weevil, *Sitophilus granarius* L. In: Highley E., Wright, E. J., Banks, H. J., Champ, B. R. (Eds). *Proceedings of the 6th International Working Conference on Stored Product Protection Volume 2*, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, Oxon, pp. 650-659.
- Jacobson, R. and Pinniger, D. B. (1982). Eradication of *Oryzaephilus surinamensis* from a farm store. *International Pest Control*, 24: 68-74.
- Jaronski, S.T. (1997). New paradigms in formulating mycoinsecticides. In: Goss, R.G., Hopkinson M.J., and Collins, H.M. (eds). *Pesticide Formulations and Application Systems*. Philadelphia, ASTM. 17: 99-112.
- Jones, K. A. and Burges, D. (1998). Technology of Formulation and Application. In: Burges, H.D. (Ed.). *Formulation of Microbial Biopesticides: Beneficial Microorganisms, Nematodes and Seed Treatments*, Dordrecht, Kluwer Academic Publishers: pp 7-30.
- Jeffs, L.B., Xavier, I.J., Matai, R.E. and Khachatourians, G.G. (1999). Relationships between fungal spore morphologies and surface properties for entomopathogenic members of the genera *Beauveria*, *Metarhizium*, *Paecilomyces*, *Tolypocladium* and *Verticillium*. *Canadian Journal of Microbiology*, 45: 936-948.
- Jilani, G. and Saxena, R.C. (1990). Repellent and feeding deterrent effect of turmeric oil, sweetflag oil, neem oil and a neem based insecticide against lesser grain borer (Coleoptera: Bostrychidae). *Journal of Economic Entomology*, 83: 629-634.
- Jilani, G. and Su, H.C.F. (1983). Laboratory studies on several plant materials as insect repellants for protection of cereal grains. *Journal of Economic Entomology*, 76: 154-157.
- Jilani, G., Saxena, R.C. and Rueda, B.P. (1988). Repellent and growth-inhibiting effects of tumeric oil, sweetflag oil, neem oil and 'Margosan-O' on red flour beetle (Coleoptera: Tenebrionidae). *Journal of Economic Entomology*, 81: 1226-1230.
- Kavallieratos, N.G., Athanassiou, C.G., Michalaki, M.P., Batta, Y.A., Rigatos, H.A., Pashalidou, F.G., Balotis, G.N., Tomanovic, Z. and Vayias, B.J. (2006). Effect of the combined use of *Metarhizium anisopliae* (Metschnikoff) Sorokin and diatomaceous earth for the control of three stored-product beetle species. *Crop Protection*, 25: 1087-1094.
- Khan, M.A. (1983). Further investigations on repellency of chemical compounds to stored product insect pests. *Zeitschrift fur Angewandte Zoologie*, 70: 369-381.

- Laudani, H. and Swank, G. (1954). A laboratory apparatus for determining repellency of pyrethrum when applied to grain. *Journal of Economic Entomology*, 47: 1104-1107
- Laudani, H., Davis, D.F. and Swank, G.R. (1955). A laboratory method of evaluation the repellency of treated paper to stored-product insects. *TAPPI*, 38: 336-341.
- Liscombe, E.A.R. and Watters, F.L. (1962). Insect and mite infestations in empty granaries in the Prairie Provinces. *Canadian Entomologist*, 94: 433-441.
- Lord, J.C. (2001). Desiccant dusts synergise the effect of *Beauveria bassiana* (Hyphomycetes: Moniliales) on stored-grain beetles. *Journal of Economic Entomology*, 94: 367-372.
- Lord, J.C. (2005). Low humidity, moderate temperature and desiccant dust favor efficacy of *Beauveria bassiana* (Hyphomycetes: Moniliales) for the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bruchidae). *Biological Control*, 34: 180-186.
- Lord, J.C. (2007a). Desiccation increases the efficacy of *Beauveria bassiana* for stored-grain pest insect control. *Journal of Stored Products Research*, 43: 535-539.
- Lord, J.C. (2007b). Enhanced efficacy of *Beauveria bassiana* for red flour beetle with reduced moisture. *Journal of Economic Entomology*, 100: 1071-1074.
- Loshciavo, S.R. (1952). A study of some food preferences of *Tribolium confusum* Duv. *Cereal Chemistry*, 29: 91-107.
- Lynch, J.M., Harper, S.H.T. and Sladdin, M. (1981). Alleviation, by a formulation containing calcium peroxide and lime, of microbial inhibition of cereal seedling establishment. *Current Microbiology*, 5: 27-30.
- Magan, N. (2001). Physiological Approaches to Improving the Ecological Fitness of Fungal Bicontrol [sic] Agents. In: T. M. Butt, C. Jackson and N. Magan (Eds). *Fungi As Biocontrol Agents: Progress, Problems and Potential*, CAB International, Wallingford UK, 239-251.
- Matthews, G.A. (1979). *Pesticide Application Methods*. Longman, London, 334 pp.
- McDonald, L.L., Guy, R.H. and Speirs, R.D. (1970). Preliminary evaluation of new candidate materials as toxicants, repellents and attractants against stored product insects. *Marketing Research Report, No. 882*. Agricultural Research Service, USDA, Washington, 8pp.
- McGonigle, D.F., and Jackson, C.W. (2002). Effect of surface material on electrostatic charging of houseflies (*Musca domestica* L.). *Pest Management Science*, 58: 374-380.
- McGonigle, D.F., Jackson, C.W. and Davidson, J.L. (2002). Triboelectrification of houseflies, *Musca domestica*, L. walking on synthetic dielectric surfaces. *Journal of Electrostatics* 54: 167-177.
- McLaughlin, A. (1994). Laboratory trials on desiccant dust insecticides. In: Highley, E., Wright, E.J., Banks, H.J., Champ, B.R. (Eds), *Proceedings of the 6th International Working Conference on Stored Product Protection*, Canberra, Australia 1994, pp. 638-645.
- Meikle, W.G., Cherry, A.J., Holst, N., Hounna, B. and Markham, R.H. (2001). The effects of an entomopathogenic fungus, *Beauveria bassiana* (Balsamo) vuillemin (Hyphomycetes), on *Prostephanus truncatus* (Horn) (Col.: Bostrichidae), *Sitophilus zeamais* Motschulsky (Col.:

- Curculionidae), and grain losses in stored maize in the Benin Republic. *Journal of Invertebrate Pathology*, 77: 198-205.
- Meikle, W.G., Mercadier, G., Holst, N., Nansen, C. and Girod, V. (2007). Duration and spread of an entomopathogenic fungus, *Beauveria bassiana* (Deuteromycota: Hyphomycetes), used to treat varroa mites (Acari: Varroidae) in honey bee (Hymenoptera: Apidae) hives. *Journal of Economic Entomology*, 100: 1-10.
- Michalaki, M.P., Athanassiou, C.G., Kavallieratos, N.G., Batta, Y.A. and Balotis, G.N. (2006). Effectiveness of *Metarhizium anisopliae* (Metschnikoff) Sorokin applied alone or in combination with diatomaceous earth against *Tribolium confusum* Du Val larvae: influence of temperature, relative humidity and type of commodity. *Crop Protection*, 25: 418-425.
- Mohan, S. and Fields, P.G. (2002). A simple technique to assess compounds that are repellent or attractant to stored-product insects. *Journal of Stored Products Research*, 38: 23-31.
- Moino, A., Alves, S.B. and Pereira, R.M. (1998). Efficacy of *Beauveria bassiana* Vuillemin isolates for control of stored grain pests. *Journal of Applied Entomology*, 122: 301-305.
- Mordue, A.J. and Blackwell, A. (1993). Azadirachtin: an update. *Journal of Insect Physiology*, 39: 903-924.
- Mullen, M.A. and Pederson, J.R. (2000). Sanitation and exclusion. In: Subramanyam, B., Hagstrum, D.W. (Eds). *Alternatives to Pesticides in Stored-Product IPM*. Kluwer Academic Publishers, Massachusetts, pp. 29-50.
- Nakajima, S, Okamura, A, Takeda, T, Sugawara, K, Tateishi, M, Iwasa, J, Baba, N. (1997). Synthesis of 13-oxo-(Z)-9-octadecenoic acid and 15-oxo-(Z)-11-eicosenoic acid, arrestants of *Oryzaephilus surinamensis* L. *Bioscience Biotechnology and Biochemistry*, 61: 551-552.
- Nansen, C., Barton, L.E.E. and Nansen, M. (2007). Uptake, retention, and repellency of a potential carrier of active ingredients in crack and crevice treatments for store-grain beetles. *Journal of Stored Products Research*, 43: 417-424.
- Nansen, C., MacDonald, K.M., Rogers, C.D., Thomas, M., Poppy, G.M. and Baxter, I.H. (2007). Effects of sex pheromone in electrostatic powder on mating behaviour by *Lobesia botrana* males. *Journal of Applied Entomology*, 131: 303-310.
- Neves, P.M.O.J. and Alves, S.B. (2004). External events related to the infection process of *Cornitermes cumulans* (Kollar) (Isoptera: Termitidae) by the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *Neotropical Entomology*, 33: 51-56.
- Obeng-Ofori, D. and Reichmuth, Ch. (1997). Bioactivity of eugenol, a major component of essential oil of *Ocimum suave* (Wild.) against four species of stored-product Coleoptera. *International Journal of Pest Management*, 43: 89-94.
- Papachristos, D.P. and Stamopoulos, D.C. (2002). Repellent, toxic and reproduction inhibitory effects of essential oil vapours on *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). *Journal of Stored Products Research*, 38: 117-128.

- Pappas, P.W. and Morrison, S.E. (1995). Benzoquinones for the beetles, *Tribolium castaneum* and *Tribolium confusum*. *Preparative Biochemistry*, 25: 155-168.
- Pesticides Safety Directorate (2008). Assessment of the impact on crop protection in the UK of the 'cut-off criteria' and substitution provisions in the proposed Regulation of the European Parliament and of the Council concerning the placing of plant protection products in the market. Pesticides Safety Directorate, UK, 46 pp.
- Pekrul, S. and Gula, E.A. (1979). Mode of infection of the corn earworm (*Heliothis zea*) by *Beauveria bassiana* as revealed by scanning electron microscopy. *Journal of Invertebrate Pathology*, 34: 238-247.
- Pinniger, D.B. (1974). A laboratory simulation of residual populations of stored product pests and an assessment of their susceptibility to a contact insecticide. *Journal of Stored Products Research*, 10: 217-223.
- Prendeville, H.R. and Stevens, L. (2002). Microbe inhibition by *Tribolium* flour beetles varies with beetle species, strain, sex and microbe group. *Journal of Chemical Ecology*, 28: 1183-1190.
- Prickett, A.J. and Ratcliffe, C.A. (1977). The behaviour of *Tribolium castaneum* (Herbst.) and *Sitophilus granarius* (L.) in the presence of insecticide-treated surfaces. *Journal of Stored Products Research*, 13: 145-148.
- Quesdad-Moraga, E. and Vey, A. (2004). Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana*. *Mycological Research*, 108: 441-452.
- Rice, W.C. and Cogburn, R.R. (1999). Activity of the entomopathogenic fungus *Beauveria bassiana* against three coleopteran pests of stored grain. *Journal of Economic Entomology*, 92: 691-694.
- Rigaux, M., Haubruge, E. and Fields, P. (2001). Mechanism for tolerance to diatomaceous earth between strains of *Tribolium castaneum*. *Entomologia Experimentalis et Applicata*, 101: 33-39.
- Roberts, D.W. (1981). Toxins of entomopathogenic fungi. In: Burges, H.D. (Ed). *Microbial control of pests and plant diseases 1970-1980*, Academic Press, London, UK, pp 441-464.
- Saxena, R.C., Jilani, G. and Kareem, A.A. (1989). Effects of neem on stored grain insects. In: Jacobson, M. (Ed.). *Focus on Phytochemical Pesticides Vol. 1. The Neem Tree*. CRC Press, Boca Raton. pp. 97-111.
- Schimazu, M. and Sato, H. (1996). Media for selective isolation of an entomogenous fungus *Beauveria bassiana* (Deuteromycotina:Hyphomycetes). *Applied Entomology and Zoology*, 31: 291-298.
- Searle, T. and Doberski, J. (1984). An investigation of the entomogenous fungus *Beauveria bassiana* as a potential biological control agent of *Oryzaephilus surinamensis*. *Journal of Stored Products Research*, 20: 17-23.
- Seeger, C., Längle, T., Pernfuss, B., Stuppner, H. and Strasser, H. (2005). High-performance liquid chromatography-diode array detection assay for the detection and quantification of the

- Beauveria* metabolite oosporein from potato tubers. *Journal of Chromatography A*, 1092: 254-257.
- Sheeba, G., Seshadri, S., Raja, N., Janarthanan, S. and Ignacimuthu, S. (2001). Efficacy of *Beauveria bassiana* for the control of the rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). *Applied Entomology and Zoology*, 36: 117-120.
- Smith, S.M., Moore, D., Oduor, G.I., Wright, D.J., Chandi, E.A. and Agano, J.O. (2006). Effect of wood ash and conidia of *Beauveria bassiana* (Balsamo) Vuillemin on mortality of *Prostephanus truncatus* (Horn). *Journal of Stored Products Research*, 42: 357-366.
- St. Leger, R.J. (1991). Integument as a barrier to microbial infections. In: Binnington, K. and Retnakaran, A. (Eds), *Physiology of the insect epidermis*, CSIRO, Australia, pp 284-306.
- St. Leger, R.J. (1993). Biology and mechanisms of insect-cuticle invasion by deuteromycete fungal pathogens. *Parasites and pathogens of insects*, Vol. 2: Pathogens pp 211-229.
- Strasser, H., Abendstein, D., Stuppner, H. and Butt, T.M. (2000a). Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. *Mycological Research*, 104: 1227-1233.
- Strasser, H., Vey, A. and Butt, T.M. (2000b). Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? *Biocontrol, Science and Technology*, 10: 717-735.
- Su, H.C.F. (1986). Laboratory evaluation of the toxicity and repellency of coriander seed to four species of stored product insects. *Journal of Entomological Science*, 21: 169-174.
- Subramanyam, B., Swanson, C.L., Madamanchi, N. and Norwood, S. (1994). Effectiveness of *Insecto*, a new diatomaceous earth formulation in suppressing several stored-grain insect species. In: Highley E., Wright, E.J., Banks, H.J. and Champ, B.R. (Eds). *Proceeding of the 6th International Working Conference Stored-product Protection*, Canberra, Australia CAB International, Wallingford, U.K. pp. 650-659.
- Tapondjou, A.L., Adler, C., Fontem, D.A., Bouda, H. and Reichmuth, C. (2005). Bioactivities of cymol and essential oils of *Cupressus sempervirens* and *Eucalyptus saligna* against *Sitophilus zeamais* Motschulsky and *Tribolium confusum* du Val. *Journal of Stored Products Research*, 41: 91-102.
- Trematerra, P. and Sciarretta, A. (2002). Activity of chilli, *Capsicum annum* L. var. *acuminatum*, on stored product insects *Oryzaephilus surinamensis* (L.), *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst). *Bulletin of OILB/SROP. International Organisation for biological and integrated control of noxious animals and plants (OIBC/OILB), West Palaearctic Regional Section (WPRS/SROP), Dijon, France 2002*. 25: 177-182.
- Trematerra P., Fontana, F., Mancini, M. and Sciarretta A. (1999). Influence of intact and damaged cereal kernels on the behaviour of rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). *Journal of Stored Products Research*, 35: 265–276.

- Unruh, L.M., Xu, R. and Kramer, K.J. (1998). Benzoquinone levels as a function of age and gender of the red flour beetle, *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, 28: 969-977.
- Vassilakos, T.N., Athanassoïu, C.G., Kavallieratos, N.G. and Vayias, B.J. (2006). Influence of temperature on the insecticidal effect of *Beauveria bassiana* in combination with diatomaceous earth against *Rhyzopertha dominica* and *Sitophilus oryzae* on stored wheat. *Biological Control*, 38: 270-281.
- Verheggen, F., Ryne, C., Olsson, P.-O.C., Arnaud, L., Lognay, G., Högberg, H.-E., Persson, D., Haubruge, E. and Löfstedt, C. (2007). Electrophysiological and behavioural activity of secondary metabolites in the confused flour beetle, *Tribolium confusum*. *Journal of Chemical Ecology*, 33: 525-539.
- Vey, A., Hoagland, R.E. and Butt, T.M. (2001). Toxic metabolites of fungal biocontrol agents. In: Butt, T. M., Jackson, C. and Magan, N (Eds). *Fungi as Biocontrol Agents*, CABI International, Wallingford, UK p 311-346.
- Watson, E. and Barson, G. (1996). A laboratory assessment of the behavioural responses of three strains of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) to three insecticides and the insect repellent N, N-diethyl-m-toluamide. *Journal of Stored Products Research*, 32: 59-67.
- Weaver, D.K. and Subramanyam, B. (2000). Botanicals. In: Subramanyam, B., Hagstrum, D.W. (Eds). *Alternatives to Pesticides in Stored-Product IPM*, Kluwer Academic Publishers, Massachusetts, pp. 303-320.
- Whalon, M.E., Mota-Sanchez, D., Hollingworth, R.M. and Duynslager, L. (2008). The Arthropod Pesticide Resistance Database. <http://www.pesticideresistance.org>
- White, G.D., Berndt, W.L. and Wilson, J.L. (1975). Evaluating diatomaceous earth, silica-aerogel dusts and malathion to protect stored what from insects. Marketing Research Report, Agricultural Research Service, USDA, 1038: 18.
- Wildey, K.B. (1984). Studies on the effects of repellency on the efficacy of residual insecticides against resistant and susceptible strains of *Tribolium castaneum*. PhD Thesis, University of Reading, 137pp.
- Wright, S.P. and Carruthers, R.I. (1999). Production, delivery and use of mycoinsecticides for control of insect pests on field crops. In: F.R. Hall and J.J. Menn (eds). *Methods in Biotechnology*, vol. 5: *Biopesticides: Use and Delivery*. Totowa, NJ, Humana Press Inc.: 233-269.
- Wright, S.P., Jackson, M.A. and Kock, S.L. (2001). Production, stabilization and formulation of fungal biocontrol agents. In: Butt, T.M., Jackson, C. and Magan, N. (Eds). *Fungi as Biocontrol Agents: Progress, Problems and Potential*, CABI International, Wallingford, UK, 253-287.

- Wright, E.J. (1990). A trapping method to evaluate efficacy of a structural treatment in empty silos. In: Fleurat-Lessard, F., Ducom, P., (Eds). Proceedings of the 6th International Working Conference on Stored Product Protection, Bordeaux, France. Institut National de la Recherche Agronomique, INRA, Paris France, pp. 1455-1463.
- Xie, Y.S., Fields, P.G. and Isman, M.B. (1995). Repellency and toxicity of azadirachtin and neem concentrates to three stored-product beetles. *Journal of Economic Entomology*, 88: 1024-1031.
- Yezerki, A., Gilmore, T.P. and Stevens, L. (2000). Variation in the production and distribution of substituted benzoquinone compounds among genetic strains of the confused flour beetle, *Tribolium confusum*. *Physiological and Biochemical Zoology*, 73: 192-199.
- Yezerki, A., Ciccone, C., Rozitski, J. and Volingavage, B. (2007). The effects of a naturally produced benzoquinone on microbes common to flour. *Journal of Chemical Ecology*, 33: 1217-1225.
- Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, 17: 553-596.

APPENDIX I: DATA REQUIREMENTS FOR ANNEX II

1. IDENTITY OF THE MICROORGANISM

Applicant

Producer

Name of the species and strain characterisation

Specification of the material used for manufacturing the formulated product

2. BIOLOGICAL PROPERTIES OF THE MICROORGANISM

History of the microorganism and its uses and natural occurrence and geographical distribution

Information on the target organism(s)

Host specificity range and effects on species other than the target harmful organism

Development stages / life cycle of the micro-organism

Ineffectiveness, dispersal and colonisation ability

Relationships to known plant or animal or human pathogens

Genetic stability and factors affecting it

Information on the production of metabolites, especially toxins

Antibiotics and other anti-microbial agent

3. FURTHER INFORMATION ON THE MICROORGANISM

Introduction

Function

Field of use envisaged

Crops or products protected or treated

Method of production and quality control

Information on occurrence or the possible occurrence of the development of resistance of the target organism

Methods to prevent loss of virulence of seed stock of the microorganism

Recommended methods and precautions concerning handling, storage, transport or fire

Procedures for destruction or decontamination

Measures in case of an accident

4. ANALYTICAL METHODS

Introduction

Methods for the analysis of the microorganism as manufactured

Methods to determine and quantify residues (viable or non-viable)

5. EFFECTS ON HUMAN HEALTH

Introduction

TIER I

Basic information.

Medical data

Medical surveillance on manufacturing plant personnel

Sensitisation / allergy observations, if appropriate

Direct observation, e.g. clinical cases

Basic studies

Sensitisation

Acute toxicity pathogenicity and infectiveness

 Acute oral toxicity, pathogenicity and infectiveness

 Acute inhalation toxicity, pathogenicity and infectiveness

 Intraperitoneal / subcutaneous single dose

Genotoxicity testing

 In vitro studies

Cell culture study

Information on the short-term toxicity and pathogenicity

 Health affects after repeated inhalatory exposure

Proposed treatment: first aid measures, medical treatment.

TIER II

Specific toxicity, pathogenicity and infectiveness studies

In vivo studies in somatic cells

Genotoxicity – in vivo studies in germ cells

Summary of mammalian toxicity, pathogenicity and infectiveness and overall evaluation

6. RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED

Introduction

Persistence and likelihood of multiplication in or on crops, feedingstuffs or foodstuffs

Further information required

 Non-viable residues

 Viable residues

Summary and evaluation of residue behaviour resulting from data and submitted under points 6.1 and 6.2

7 FATE AND BEHAVIOUR IN THE ENVIRONMENT

Introduction

Persistence and multiplication

Soil

Water

Air

Mobility

8. EFFECTS ON NON-TARGET ORGANISMS

Introduction

Effects on birds

Effects on aquatic organisms

Effects on bees

Effects on arthropods, other than bees

Effects on earthworms

Effects on non-target soil micro-organisms.

Additional studies

9. SUMMARY AND EVALUATION OF ENVIRONMENTAL IMPACT

APPENDIX II DATA REQUIREMENTS FOR ANNEX III

1. IDENTITY OF THE PLANT PROTECTION PRODUCT

Applicant

Manufacturer of the preparation and the micro-organism(s)

Trade name or proposed trade name and manufacturer's development code number of the preparation if appropriate.

Detailed quantitative and qualitative information on the on the composition of the preparation

Physical state and nature of the preparation

Function

2. PHYSICAL, CHEMICAL AND TECHNICAL PROPERTIES OF THE PLANT PROTECTION PRODUCT

Appearance (colour and odour)

Storage stability and shelf-life

Explosivity and oxidising properties

Flashpoint and other indications of flammability or spontaneous ignition

Acidity, alkalinity and if necessary pH value

Viscosity and surface tension

Technical characteristics of the plant protection product (as far as applicable)

Wettability

Persistent forming

Suspensibility and suspension stability

Dry sieve test and wet sieve test

Particle size distribution, content of dust, attrition and friability

Emulsifiability, re-emulsifiability and emulsion stability

Flowability, pourability and dustability

Physical chemical and biological compatibility with other products including plant protection product, with which its use is to be authorised

Adherence and distribution to seeds

3. DATA ON APPLICATION

Fields of use envisaged

Mode of action

Details of intended use

Application rate

Content of micro-organisms in material used

Method of application

Number and timing of applications and duration of protection

Necessary waiting periods or other precautions to avoid phytopathogenic effects on succeeding crops

Proposed instructions for use

4. FURTHER INFORMATION ON THE PLANT PROTECTION PRODUCT

Packaging and compatibility of the preparation with proposed packaging materials

Procedures for cleaning application equipment

Re-entry periods, necessary waiting periods or other precautions to protect man livestock and the environment

Recommended methods and precautions concerning handling, storage, transport or fire

Measures in case of an accident

Procedures for destruction or decontamination of the plant protection product and its packaging

5. ANALYTICAL METHODS

Introduction

Methods for the analysis of the preparation

Methods, to determine and quantify residues

6. EFFICACY DATA (According to Dir. 93/71/EEC of 27/7/93)

Introduction

A technical dossier is required for evaluating foreseeable risks, whether immediate or delayed and to evaluate the benefits. Basically the claims on the label should be confirmed by the trials.

(Comment SDvH: For biologicals the efficacy requirements are not as stringent as for chemicals)

Trials should be conducted according to standard (e.g. EPPO, US EPA) guidelines under GEP by accredited organisations.

Per country normally a minimum of 8 trials over 2 years are required. Trials in other countries in the same (climatic, soil type etc.) zone may replace some trials for a country (Comment SDvH: This latter derogation is different from country to country; one should check with the authorities of a particular country where the registration application is made).

Any trial should contain also a comparison product and an untreated plot.

Preliminary tests

Efficacy trials

Occurrence or the possible occurrence of resistance

Effects on yield, quantitatively and qualitatively

Effects of phytotoxicity

Observations on undesirable or unintended side-effects, such as follow crops

Effects on beneficial or other non-target species

7. EFFECTS ON HUMAN HEALTH

Basic, acute toxicity studies

Acute oral toxicity

Acute inhalation toxicity

Acute percutaneous toxicity

Additional, acute toxicity studies

Skin indication

Eye irritation

Skin sensitisation

Data on exposure

Available toxicological data relating to non-active substances

Supplementary studies for combinations of plant protection products.

Summary and evaluation of health affects.

8. RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED

The same requirements as for Annex II section 6.

9. FATE AND BEHAVIOUR IN THE ENVIRONMENT

The same requirements as for ANNEX II section 7

10 EFFECTS ON NO-TARGET ORGANISMS

Effects on birds

Effects on aquatic organisms

Effects on bees

Effects on arthropods, other than bees

Effect on earthworms

Effects on soil micro-organisms

Additional studies

11. SUMMARY AND EVALUATION OF ENVIRONMENTAL IMPACT

APPENDIX III. SPRAYER CALIBRATION FOR PILOT SCALE TRIAL 2

Introduction

Pilot scale trial 1 used an ULVA sprayer that delivered consistent droplet spectra into arenas. However, this technology relies on the ability to walk whilst applying, as the swath of the sprayer is circular and droplets are created from a centrifugal spinning disc. This method was suitable for application during the first pilot scale trial as arenas with identical treatments were situated next to each other for the comparison of adding insects before and 24h after spraying. However, the number of conidia delivered to each arena was less than expected. The ULVA sprayer was not used in trial 2, as trial arenas with the same application dose/method were not situated adjacent to each other and with the ring shaped swath it would not be possible to deposit a known amount of conidia within the trial arena. Arenas for pilot scale trial 2 were 50 cm in diameter and the desired concentrations of conidia to be delivered per arena were either 1×10^{10} or 5×10^9 .

Plant sprayers (Hozelock Spraymist) were examined to see if it would be possible to calibrate them to produce a consistent reproducible method of delivering conidia into the trial arenas. They were calibrated by monitoring the consistency of the spray volume and the number of conidia delivered in a certain number of sprays. By adjusting the strength of the stock solution and the position of the spray nozzle reliable results were achieved.

Materials and Methods

Amount deposited with blank oil

Initially a blank oil (50:50 Shellsol T:Light White Mineral oil) was tested with the sprayer to assess how much oil was delivered. The amount of oil delivered was measured in a 10 ml measuring cylinder.

Amount deposited with test formulations

The relevant weight of conidia was suspended in the appropriate amount of carrier oil (50:50 mix of Shellsol T and Light White mineral oil) and the concentration of the formulations was calculated by serial dilution to give a solution with a 10^{-3} dilution. The concentration of this dilution was then counted on a haemocytometer. Each formulation was then assessed by passing it through the sprayer. Testing was carried out by spraying the formulation into pre-weighed universals. The weight of spray delivered and the concentration of the spray were measured. Various adjustments were made to the spray technique, sprayer settings and formulation concentration in order to obtain reliable spray concentrations and amounts delivered. The calibration was an iterative process and details of amendments to formulation concentration and spray technique are covered in the results section.

Results

Amount deposited with blank oil

Spray coverage of an area roughly 30 x 50 cm was assessed and it was found that two sprays covered the area sufficiently with blank oil. Table 1 shows the amount of oil delivered with 5 sprays of the plant sprayer. It was noted that by reusing the measuring cylinder a residue of oil was left; therefore, subsequent methods used a pre-weighed universal.

Table 1. Amount of oil delivered by plant sprayer 01/2009

Rep	No. Sprays	Volume Delivered	Average per spray (ml)	Overall Average
1	5	5.6	1.12	1.19ml
2	5	5.8	1.16	
3	5	6.2	1.24	
4	5	6.2	1.24	
5	5	6.2	1.24	
6	5	5.8	1.16	

Amount deposited with test formulations

The first formulation prepared was 4.2×10^9 per ml, which according to the average amount sprayed with blank oil, and accounting for two sprays from the sprayer, should deliver 1×10^{10} conidia. On counting the concentration of conidia delivered, it was found that only 6.1×10^9 was delivered per 2 sprays. It was assumed that conidia were adhering to the plastic of the sprayer; therefore, concentrations of conidia were increased to compensate for this.

The concentration of the formulation was adjusted accordingly to give a concentration of 8.2×10^9 conidia/ml. This formulation was prepared and tested in sprayer number 01/2009. Ten priming sprays were carried out before the formulation was sprayed in to the pre-weighed tubes. During these priming sprays, it was noted that spray consistency was different between sprays and it was suspected that the nozzle was blocking due to the higher concentration of formulation. Therefore, it was decided that diluting the formulation and spraying with three sprays instead of two would produce a more consistent result. The formulation was diluted and the concentration was recalculated at 4.1×10^9 per ml. Three sprays were applied to ten separate pre-weighed 10 ml universals. These universals were reweighed and the weight of formulation applied calculated. From the spray and the weight data, the concentration of conidia applied in 3 sprays was calculated (Table 2).

Table 2. Concentration of spray delivered from formulation stock 4.1×10^9 per ml using sprayer 01/2009.

Rep	Weight of spray	Concentration delivered in 3 sprays	AVERAGE	SE
1	3.3042	1.8×10^{10}	1.8×10^{10}	1.4×10^9
2	3.2824	2.1×10^{10}		
3	2.9076	1.8×10^{10}		
4	3.3637	2.3×10^{10}		
5	3.3229	2.1×10^{10}		
6	3.2076	1.3×10^{10}		
7	3.3915	2.4×10^{10}		
8	3.4106	1.9×10^{10}		
9	2.658	1.1×10^{10}		
10	3.3322	1.4×10^{10}		

Further testing used different nozzle positions and spray tank mixing techniques in order to improve the consistency of the spray (Table 3). It was found that the best position for the nozzle was half a turn back from the tightest position and that by swirling the sprayer before each spray, a consistent amount was delivered. In order to test this fully, a new formulation was prepared and 20-24 calibration sprays were made. All the tubes were weighed to check the consistency of delivery and a random sample were counted in order to check the concentration of conidia delivered (Table 4). The same protocol was used for sprayer number 02/2009 which was designated to deliver the 5×10^9 treatment. However, the spray concentration for this was much lower so therefore the work to optimize sprayer nozzle positioning was not needed. The results for sprayer number 02/2009 can be seen in Table 5. The starting concentration for the calibration of sprayer number 01/2009 was 5.5×10^9 conidia per ml and for sprayer number 02/2009 was 1.9×10^9 conidia per ml.

Table 3. Concentration of spray and amount delivered with different nozzle positions and spray tank mixing. Replicates 11-15 were not counted as the sprays were 'light'. Stock concentration was 4.1×10^9 conidia per ml.

Rep	Nozzle position	Technique	Weight of spray (g)	Total concentration delivered in 3 sprays
11	Nozzle at tightest	1 swirl before 3 sprays	2.5012	Not counted
12	Nozzle at tightest	1 swirl before 3 sprays	2.3241	Not counted
13	Nozzle at tightest	1 swirl before 3 sprays	1.4187	Not counted
14	½ turn back from tightest	1 swirl before 3 sprays	2.7567	Not counted
15	½ turn back from tightest	1 swirl before 3 sprays	2.5491	Not counted
16	½ turn back from tightest	1 swirl before EACH spray	3.096	1.1×10^{10}
17	½ turn back from tightest	1 swirl before EACH spray	3.3438	1.7×10^{10}
18	½ turn back from tightest	1 swirl before EACH spray	3.3317	1.9×10^{10}
19	½ turn back from tightest	1 swirl before EACH spray	3.4793	1.2×10^{10}
20	½ turn back from tightest	1 swirl before EACH spray	3.3892	1.6×10^{10}

Table 4. Calibration results for sprayer number 01/2009. Ten samples were randomly selected to determine the concentration of the spray. *weaker spray.

Rep	Weight of spray	Total concentration delivered in 3 sprays	Overall AVERAGE	SE
1	3.1236	1.8×10^{10}	2.2×10^{10}	1.9×10^9
2	2.6896	Not counted		
3	2.924	Not counted		
4	3.1539	1.9×10^{10}		
5	2.0336*	Not counted		
6	3.2914	Not counted		
7	3.3438	3.1×10^{10}		
8	2.3919*	2.2×10^{10}		
9	2.113*	Not counted		
10	3.3200	3.3×10^{10}		
11	2.9205*	Not counted		
12	3.0612	Not counted		
13	3.3671	1.9×10^{10}		
14	3.2909	Not counted		
15	2.7999*	Not counted		
16	3.4565	Not counted		
17	2.8944*	1.8×10^{10}		
18	3.3952	Not counted		
19	3.0487	2.4×10^{10}		
20	2.8088	1.3×10^{10}		
21	2.2050	Not counted		
22	2.9161	2.2×10^{10}		
23	2.4521	Not counted		
24	1.9992*	Not counted		

Table 5. Calibration results for sprayer number 02/2009. Nine random samples were taken to calculate the concentration sprayed.

Rep	Weight of spray	Total concentration delivered in 3 sprays	Overall AVERAGE	SE
1	2.9691	4.8×10^9	6.6×10^9	5.8×10^8
2	2.9209	Not counted		
3	2.9196	6.3×10^9		
4	2.7963	Not counted		
5	2.9128	Not counted		
6	2.8771	Not counted		
7	2.568	5.8×10^9		
8	2.7164	9.0×10^9		
9	3.0987	Not counted		
10	3.1643	9.7×10^9		
11	2.4666	Not counted		
12	2.5895	5.4×10^9		
13	2.3694	Not counted		
14	2.7663	Not counted		
15		Not counted		
16	2.8779	4.8×10^9		
17	2.9158	6.7×10^9		
18	2.6816	Not counted		
19	2.9227	Not counted		
20	2.9927	6.5×10^9		

Discussion

Various different techniques were employed to calibrate the sprayers. For sprayer number 01/2009, it was found that a consistent number of conidia could be delivered using three sprays of the plant sprayer, with the nozzle turned half a turn back from the tightest position. By swirling the spray tank before each spray, this also improved the consistency of results. The concentration of formulation was altered in accordance with results and the number of sprays was optimised in order to deliver the correct concentration of conidia. The final calibration for sprayer number 01/2009 (Table 4) showed that too many conidia were delivered with the spray concentration at 5.5×10^9 per ml and therefore the concentration was reduced to 4.1×10^9 per ml for the pilot scale trial to deliver 1×10^{10} conidia into the arena. For the 5×10^9 conidia/ml treatment, 1.9×10^9 conidia/ml concentration was used.