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THE POTENTIAL USE OF INSECT-SPECIFIC FUNGI TO CONTROL GRAIN STORAGE PESTS IN EMPTY GRAIN STORES

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by

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Abstract

Stored grain and grain products are at risk of infestation by insects and mites. However, some OP pesticides currently used to control these pests are being withdrawn from use and methyl bromide will be phased out for most uses by the end of 2004. Alternative approaches for the control of storage pests are needed urgently. This project aimed to collect UK isolates of naturally-occurring insect specific fungi and evaluate their use for grain store structure treatments.

Samples were collected from UK storage premises and approximately 70,000 insect cadavers were processed. Fungal isolates were established in laboratory culture. Molecular and biological characterisation of 8 isolates, identified as *Beauveria bassiana,* was completed. These and some additional isolates from the CABI Bioscience Genetic Resource Collection (GRC) were tested in a primary laboratory screen against four representative stored-food pests. The most successful isolates were mass-produced and taken forward to a secondary laboratory screen to obtain dose-response data for the four species tested previously. The most effective isolates gave mortalities close to 100% for beetles and moths, and around 75% for mites and psocids. Some healthy grain beetles also succumbed to *B. bassiana* by picking up a lethal dose of spores from dead insects showing signs of external sporulation of the fungus. The potential of PC Floor Traps for disseminating spores was also demonstrated.

The effects of temperature and relative humidity (RH) on isolate efficacy were assessed. It was found that to obtain good control, particularly of the beetles, high humidity close to 100% RH was needed for the first 24 hours of exposure. Methodologies were evaluated for assessing efficacy and persistence of formulated fungal isolates applied to various grain store building materials under UK storage conditions. Tests with the most promising formulation from the secondary screen, a 1:4 Codacide and water mix, revealed that this carrier was not suitable for use in dusty environments because it remained very sticky on non-porous surfaces. The survival and persistence of individual spores on construction materials were also assessed at a range of temperature/RH combinations; spores showed higher persistency in dry powder form than when formulated in oil.

The results of the laboratory efficacy tests showed that UK isolates of *B. bassiana* have the potential to control a range of arthropod pests in UK grain stores. However, before this potential can be realised in practice, it will be essential to conduct further research to improve spore formulation and delivery systems.

SUMMARY

Grain stored on farms and in commercial grain stores, and processed grain in mills, is at risk of infestation by insects and mites. Currently, organophosphate (OP) pesticides and the fumigant methyl bromide are used to control these pests. However, reflecting concerns over safety and the environment, some OP pesticides are being withdrawn from use and methyl bromide will be phased out for all but certain critical uses by the end of 2004. Alternative approaches for the control of storage pests are needed urgently. This project aimed to collect and identify UK isolates of naturally-occurring insect specific fungi and evaluate their use for the control of insect and mite pests of grain storage premises.

Milestones 1 and 2. Find natural populations of insect specific fungi from food stores and mills in the UK, identify fungal isolates and establish in laboratory culture.

Samples of insects, mites and debris from over 100 UK storage premises were collected by ADAS staff. Approximately 70,000 insect cadavers were processed. Fungal isolates from these samples were identified, established in laboratory culture and prepared for testing by CABI Bioscience. Identification of isolates to species was based on morphological characteristics. In addition, four techniques were used for molecular characterisation of all *Beauveria bassiana* UK isolates, and also two isolates of *B. brongniartii* and one of *Sporothrix* sp.:-

i)   Restriction analysis of the internal-transcribed-spacer (ITS) ribosomal region.
ii)   ITS sequencing.
iii)   Inter-simple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR) amplification.
iv)   Amplified fragment length polymorphism (AFLP).

Eight isolates were identified as *B. bassiana* and deposited in the CABI Bioscience Genetic Resource Collection (GRC) of fungal strains both as freeze-dried and liquid-nitrogen stocks. The ITS analyses did not show any intraspecific variation amongst the isolates. On the other hand, the results from ISSR-PCR and AFLP analyses supported each other: the results showed genetic diversity amongst the *B. bassiana* isolates from UK farms, and revealed the presence of more than one isolate within the same premises.

Biological characterisation of the new UK isolates of *B. bassiana* included measuring fungal growth and spore production at 15, 20, 25 and 30ºC to mimic a range of storage conditions.

For most isolates, the preferred temperatures for growth were 25°C and 30°C, and for sporulation were 20°C and 25°C.

Milestone 3a. Quantify and compare the activity of these fungi, together with isolates already in culture at CABI Bioscience, against storage invertebrates under environmental conditions that are appropriate to UK grain stores.

These new isolates together with some additional isolates from the CABI Bioscience culture collection were tested at CSL in a 'high-challenge' primary screen against four representative stored-food pests: an OP resistant strain of the saw-toothed grain beetle (*Oryzaephilus surinamensis*), the Mediterranean flour moth (*Ephestia kuehniella*), the black domestic psocid (*Lepinotus patruelis*), and the flour mite (*Acarus siro*). For each isolate and insect species, four replicates of 15 insects each were rolled over an agar plate containing the fungal isolate to ensure fungal conidia adhered to the exoskeleton. This unnaturally high exposure to spores was designed to provide a rapid means of identifying those isolates pathogenic to the pests. Four control replicates were also prepared using agar without fungus. Treated insects were then transferred to Petri dishes containing filter papers moistened with sterile distilled water and kept at 20ºC 70%RH. After 24 hours the insects were transferred to glass jars containing insect culture food, and mortality was assessed up to 10 days after treatment. Dead insects were examined for signs of mycosis. For the mites, filter papers treated with fungal spores were dried and used to prepare standard CSL mite recovery cells. A small amount of mite food and 20 mites were placed in each cell, five treated and five untreated control replicates for each isolate. The completed cells were left in desiccators over water at 20ºC and mortality assessed over 10 days. Several isolates gave high mortality for all the test species.

Milestone 3b. Conduct further screening of isolates.

The seven most successful isolates were mass-produced and taken forward to a secondary screen to obtain dose-response data for the four species tested previously. A Potter Tower was used to spray filter papers with spores suspended in a carrier. Oil carriers are considered to be particularly effective in dry conditions. The industrial mineral oils Ondina and Shellsol, an oilseed rape product Codacide, as well as various mixtures of oil and water, were tested. A 1:4 mix of Codacide and sterile distilled water was found to be the least toxic or repellent to the test insects. Four log doses in the concentration range $1x10^5 - 1x10^8$ conidia/ml were prepared by serial dilution, and tested against all four representative pest species. The filter papers were dried and insects confined to the treated surfaces for 24 hours in Petri dishes. The insects were then transferred to clean untreated filter papers in Petri dishes with a little food for 10 days. To ensure good control of these beetles, it was found necessary to spray them

directly at the same time as the filter papers. For the mites, small sprayed filter papers were used to prepare standard CSL mite recovery cells in which the mites were confined for 24 hours, before being transferred to clean untreated cells. Controls were treated with carrier only. Twenty insects or mites were used in each of five replicates at each concentration. Dead insects and mites were retained to confirm that death was due to mycosis.

The highest mortalities occurred at the maximum dose of 1×10^8 conidia/ml which for the best isolates gave mortalities close to 100% for the beetles and moths, and around 75% for mites and psocids. The four best isolates were also tested against a further four pest species: the grain weevil (*Sitophilus granarius*), the rust red grain beetle (*Cryptolestes ferrugineus*), the confused flour beetle (*Tribolium confusum*), an OP susceptible strain of the saw toothed grain beetle *Oryzaephilus surinamensis,* and the grainstack mite (*Tyrophagus longior*). The isolates were particularly effective against *C. ferrugineus* with mortalities in the range 86-100%.

The effects of different temperature and RH combinations on the efficacy of four of the best isolates at the highest dose of 1×10^8 conidia/ml were tested against the beetle *Oryzaephilus* and the mite *Acarus.* These tests were conducted at 15°C 80%RH and 20°C 80%RH with and without the extra period of high RH for the first 24 hours but with the spraying of beetles directly. Without extra humidity, efficacy was very poor at the lower temperature, particularly for the beetles where mortality fell from 9% at 20°C to 0% at 15°C; however, when a short period of high humidity close to 100%RH was provided for the first 24 hours of exposure, mortality rose to around 40% after 10 days at 15°C 80%RH. Some extra studies were undertaken, including the preparation of SEM photomicrographs, but these provided little additional information to help our understanding of spore uptake from treated papers.

Milestones 4 and 5. Compare mycopesticidal efficacy of top candidates with appropriate insecticidal treatments, apply the most promising isolates to pilot-scale storage structures and define their effects on storage pests under practical conditions of temperature and humidity.

Methodologies were evaluated for assessing efficacy and persistence of formulated fungal isolates applied to different types of grain store building materials under typical UK grain storage conditions. In the chosen method, standard samples of wood, steel and concrete were sprayed with fungal spores, and pest species confined to the treated surfaces. Tests with the most promising formulation from the secondary screen, a 1:4 Codacide and water mix, revealed that this carrier remained very sticky on non-porous surfaces and was not suitable for

use in dusty environments such as grain stores and flour mills. Further tests, including a comparison with a conventional insecticide, await the finding of an improved carrier.

The survival and persistence of individual spores of the most effective isolate from the earlier tests were also assessed on steel surfaces at a range of temperature/RH combinations. The results were used to develop a predictive equation for spore persistency. The results showed that at 85%RH and 20ºC, 80% viability was maintained for 35 days by conidia in an oil formulation compared to nearly 80 days in a dry powder formulation. Although a different oil formulation from the Codacide and water used above, it did demonstrate good conidial survival for five weeks. Eighty percent viability is an arbitrary figure for good efficacy of a mycopesticide. In effect, the results are equivalent to the active life of a pesticide; in this case two different formulations result in the mycopesticide being, in theory, capable of persisting for between 5 and 11 weeks, superior to that of many chemicals.

Milestone 6. Mass-produce and formulate promising isolates for use in practical-scale trials.

Small scale mass production of dry spore powder using a two-stage technique was completed for 7 of the UK *B. bassiana* isolates. This involved the preparation of a fungal culture in liquid medium, which was then used to inoculate a solid rice substrate. Spores were harvested using a CABI MycoHarvester, designed to collect particles in an optimum range of approximately 3-10µm.

Trials using 2 metre square plywood arenas were completed to validate the methodology for testing isolates under practical conditions. A knapsack sprayer was used to treat the inside walls and floor of each arena containing 500 saw-toothed grain beetles. Tiny Tag data loggers recorded temperature and humidity conditions and mortality was assessed after 2 weeks. In preliminary tests with spores in the Codacide and water carrier, many saw-toothed grain beetles died when they became stuck in the Codacide; this made it difficult to interpret the results, and again highlighted the need for an improved formulation.

Milestone 7. Investigate the effects of fungi added to CSL trapping devices used for invertebrate detection in storage facilities as a way of reducing pest infestation.

Secondary cycling studies demonstrated that healthy grain beetles can succumb to *Beauveria bassiana* by picking up a lethal dose of spores from mycosed insects. Additional tests showed

that insects will pick up a lethal dose of spores from within PC Floor Traps as a means of disseminating spores.

This project demonstrated the potential of UK isolates of *B. bassiana* to control a range of invertebrate pests in the laboratory. Preliminary trials in the CSL grain storage facility highlighted some technical problems associated with the use of fungal spores in practice. To ensure good control of pests in UK grain stores, it will be essential to find ways of increasing the uptake, germination and penetration of fungal spores into the pests, for example through improved formulation and delivery systems or through the identification of isolates that are more effective at lower temperatures and relative humidities. Nevertheless, the results on secondary cycling, dissemination from traps and efficacy generally, indicate that this approach has promise as an alternative to chemical control and should be investigated further.

INTRODUCTION

Until recently, organophosphorus (OP) pesticides have been relied upon to protect stored products from damage by invertebrate pests. For example, OPs have been the main residual pesticides used to treat the fabric and structure of buildings employed to store and process cereals. In addition, annual or biennial fumigations are often carried out in flour mills to remove persistent infestations of stored product insects and mites.

However, the concerns over pesticide residues in food, adverse effects on the environment, customer preferences for foods grown and stored organically to avoid the use of pesticides, the development of pest resistance, and the stricter requirements of pesticide registration have all acted against OPs. Governments in many countries are actively seeking to reduce their use and are encouraging the search for alternatives to conventional control methods. In the UK, the only pesticides still registered for use on storage structures with which grain might come into contact are chlorpyrifos-methyl and pirimiphos-methyl, with a final use date of 2008. In flour mills, the ban after 2004 on the use of the ozone-depleting fumigant methyl bromide in EU and other countries in response to the Montreal Treaty to reduce damage to the ozone layer, and the re-registration process involving phosphine have further heightened the urgency of finding new pest control strategies that are in keeping with the desire for a more sustainable way of safeguarding our stored foodstuffs.

Biological pest control is one option being considered, either alone or as part of an integrated pest management (IPM) strategy. This method offers the opportunity of using living

organisms that are already present naturally in the storage environment for managing pests. Although they can be slower to act than chemicals, biocontrol agents are generally safer, and have the added advantage of being able to reproduce so that the amount of control agent follows the level of the pest, building up when needed but dying out when the pest has gone. In the past, the emphasis of much of the work on biocontrol for storage pests has been on using insects and mites as predators and parasitoids (See reviews by Brower *et al.,* 1996; Karg, 1996; Schöller *et al.,* 1997; Cox and Wilkin, 1998; Haines, 1999; Hodges, 1999; Wangxi *et al.,* 1999; Zdárková and Fejt, 1999).

In spite of encouraging results from some laboratory and pilot-scale studies using predators and parasitoids, and their availability through biocontrol companies in some countries (Copping, 2001; Quarles, 2001), their adoption on a large scale has been comparatively slow. This lack of acceptance may be due to a number of reasons including limited evidence of success on a commercial scale, worries over contamination of end products by predators and parasitoids, and the need to learn new management-intensive techniques and strategies for the efficient use of these biocontrol agents (Cox and Wilkin, 1996).

Recently, more attention has been given to the use of micro-organisms as control agents in the storage environment (Burges, 1981; Dales, 1994; Cox and Wilkin, 1996). Groups of micro-organisms that have been considered include protozoa (e.g. Khan and Selman, 1989; Ghosh and Saha, 1995), fungi (e.g. Moino *et al.,* 1998; Smith *et al.,* 1998), bacteria (e.g. McGaughey, 1986; Salama *et al.,* 1996; Hongyu *et al*., 1999), and viruses (e.g.Vail *et al*., 1993; Hunter-Fujita *et al*., 1998).

In some ways, microbial control agents may be more acceptable than the larger predators and parasitoids to store and mill managers and end users because microbial control agents can be applied using techniques similar to those for conventional pesticides. Microbial control agents also have the advantages of usually having little effect on non-target organisms including beneficial species (Vinson, 1990), and of being easier to store for longer periods than predators and parasitoids without loss of efficacy (Moore *et al*, 1995).

Their main disadvantages are the high specificity of some isolates which may not be able to control all the pest species present in one location, their slowness in action compared to conventional pesticides, and their low UV stability. However, these apparent disadvantages are less critical where the use of fungi in the storage environment is concerned because most stores are protected from UV light; it may also be possible to use a mix of different isolates,

which can attack a range of pest species (Moore *et al*., 2000). Although microbial control agents may not kill pests as rapidly as traditional insecticides, damage is still reduced quickly because many insects stop feeding and reproducing soon after being infected (Brower *et al.,* 1996). Whereas protozoa, bacteria and viruses have to be ingested to ensure infection of the insect (Bidochka *et al.,* 1997), fungi have the additional advantage of being able to penetrate the insect cuticle using incompletely understood chemical and physical interactions to reach the haemocoel.

Although worldwide there are a number of fungal preparations available commercially for the control of field, soil and glasshouse pests, including some containing *B. bassiana* or *Metarhizium anisopliae,* there appear to be none that are currently registered for use in the storage environment (Anon, 2001; Copping, 2001; Khetan, 2001; Quarles, 2001). The main aims of this project were to collect, identify and evaluate the use of UK isolates of naturally-occurring insect specific fungi for the control of invertebrate pests in the structure of grain storage premises.

MILESTONE 1. Find natural populations of insect-specific fungi from food stores and mills in the UK which are potent against a range of storage invertebrates.

Materials and methods

The ADAS input to the project centred on the collection of a wide range of stored product insects and mites from provender mills throughout England, which were then forwarded to CABI Bioscience for fungal extraction. All provender mills currently in production, including those attached to large animal production units and supplying in-house only, were identified and contact numbers and names obtained so that a good geographical spread of visits could be arranged.

Field survey visits began in early May 2000. No formal identification of species was required at this time, the main objective being to collect as large a sample of dead or alive insects and mites as possible regardless of type. Identification to species level was carried out when the samples were examined during fungal extraction at CABI Bioscience.

In order to enable the largest number of premises to be examined it was agreed that the use of bait bags would not be cost-effective as this would require a second visit for collection (previous attempts to get mill staff to collect and return bags in reply-paid envelopes had

proved unproductive, with very poor return rates recorded). During discussions with staff at CSL and CABI the collection of dead insects and mites was deemed to be as important as the collection of live samples as these may have been affected by fungal pathogens, so collection was also concentrated on examination of sievings of raw goods, sweepings, residues and the contents of industrial vacuum cleaners. More specialised extraction techniques were also being developed by CABI during this time.

After all the mills had been inspected it was agreed that the focus of collecting of insect samples during Years 2 and 3 should concentrate on farm grain stores.

Results and discussion

By the end of the first project year a total of 77 mills had been examined and samples of insects and/or debris that were likely to contain dead insects had been collected from all sites. All beetles, moths and mites found in the samples were forwarded immediately to CABI Bioscience. Initial results using standard extraction techniques for the samples failed to identify any suitable pathogens. Extra resource was allocated to allow for additional collection of stored product insects over the winter months.

Further input to the project was delayed by the Foot and Mouth Disease outbreak that prevented access to any premises, which may have potentially resulted in spread of disease. Once access to farms was freely available, samples of insects and mites were taken from 104 farm stores. A wide variety of storage types was examined ranging from modern floor stores, to interior and exterior bin storage, and floor stores in old stone barns. Samples of living and dead insects along with debris that might contain dead insect bodies were collected and sent to CABI for examination.

During year 4, although milestone 1 had been met, samples from another 55 sources, including those not previously targeted, such as maltings and poultry feed stores, were collected for examination by CABI.

MILESTONE 2. Identify the fungal isolates and establish laboratory cultures of appropriate fungi found during milestone 1.

Methods and materials

i) Isolation and identification.

The insect cadavers sent to CABI Bioscience were initially washed in a 0.05% sterile solution of Tween 80 (BDH), then surfaced sterilised by immersion in 1% sodium hypochlorite (NaClO) for 2 minutes and rinsed twice in sterile water. Twenty to thirty sterilised insects were placed on to moist filter paper in 9-cm Petri dishes which were sealed with "parafilm" and left at room temperature until fungal growth was visible (3 to 7 days). Fungal growth resembling *Beauveria bassiana* was isolated from individual insects and streaked onto Sabouraud Dextrose (Oxoid) agar (SDA) or Potato Carrot agar (20g grated potato, 20g grated carrot, 20g agar Oxoid no.3, 1L tap water) plates with antibiotics (60mg/L chloramphenicol and 60mg/L penicillin) and incubated at 25°C. Individual colonies resembling *B. bassiana* were repeatedly streaked onto fresh plates until pure cultures were obtained. Pure cultures were verified as being *B. bassiana* by the CABI Identification Service.

All *B. bassiana* isolates were held in the CABI Bioscience Genetic Resource Collection of fungal strains both as freeze-dried and liquid-nitrogen stocks. These stocks were re-activated by addition of sterile water and cultured on SDA or Potato Carrot agar plates incubated at 25°C. Fully sporulated culture plates were kept in cold storage (4°C) for immediate accessibility.

ii) Biological characterisation.

Biological characterization of isolates was based on growth and sporulation at different temperatures. To measure growth, 10µl of spore suspension ($10^6 – 10^7$ spores/ml) were inoculated onto SDA plates (5 replicates) for each temperature: 15°C, 20°C, 25°C and 30°C. The extent of growth was then measured (mm) from the edge of the central inoculation point towards the periphery of each plate (along crosslines) at daily intervals up to 21 days. The plates were placed randomly in the incubators after each assessment.

To measure sporulation, five 6mm plugs were cut out of each growth plate remaining on day 21; some plates were lost due to contamination. All 5 plugs, 1 taken from the centre and 4 round the edge in a cross formation, were placed in 5ml of sterile 0.05% Tween 80. After vigorous shaking, a 20µl aliquot from this spore suspension was placed on a bright-line haemocytometer and spore counts were assessed using a light microscope. Using the haemocytometer counts, mean spore yield per plug values were calculated, providing a comparative measure of yield per unit area of media for each of the isolates tested.

iii) Molecular characterisation.

In addition, four techniques were used for molecular characterisation of all *B. bassiana* UK isolates, and also two isolates of *B. brongniartii* and one of *Sporothrix* sp.: -

- Restriction analysis of the internal-transcribed-spacer (ITS) ribosomal region.
- ITS sequencing.
- Inter-simple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR) amplification.
- Amplified fragment length polymorphism (AFLP).

For DNA extraction purposes conidia were resuspended in 0.05 % Tween 80 up to 10^7-10^8 conidia/ml. From this suspension 20 μl were inoculated onto PCA or Malt Extract Agar (MA) (20 g toffee barley malt extract, 20 g agar Oxoid no.3, 1 L tap water, pH 6.5) 5 cm plates, and the plates were incubated at 25°C. After 2-3 days incubation, the mycelium was harvested and resuspended in 600 μl sterile TE buffer (1mM EDTA, 10mM Tris-HCl, pH 8) and kept at –20°C until use. After defrosting, the mycelium suspension was homogenized using conical grinders and genomic DNA from all isolates were extracted using the QIAmp® DNA Mini Kit (QIAGEN, Germany), with the following modifications: incubation with 400 units of lyticase for 2 h at 30°C, then an overnight incubation with protease. The DNA was eluted in 100 μl of sterile water. The final DNA concentration was in the range of 20–200 ng μl $^{-1}$ measured using the spectrophotometer GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech) (Aquino de Muro *et al*. 2003). The DNA extracts were stored at –20°C.

The ribosomal internally transcribed spacer (ITS) regions ITS1 and ITS2 flanking the 5.8S subunit were amplified with the following pair of primers: ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990). The primers were synthesised by Amersham Pharmacia Biotech. PCR conditions and RFLP analysis were as described in Aquino de Muro *et al.* (2003). The ITS fragments for cloning and automated ABI sequencing from 20 isolates of *B. bassiana,* 1 isolate of *Beauveria* sp. and 1 isolate of *B. brongniartii* (indicated by asterisks in Table 1) were purified using Promega Wizard PCR Preps DNA Purification System. The sequence alignment and a Neighbour-Joining dendrogram were generated using Clustal W (Thompson *et al.* 1994, Aiyar, A. 2000).

For the ISSR-PCR two sequence repeats $(CCA)_5$ and $(TGT)_5$ were amplified with 100 pmol of the respective primers 5'-DD CCA CCA CCA CCA CCA- 3' or 5'-VHV TGT TGT TGT TGT TGT-3' in a 20 µl PCR reaction mixture, which consisted of: 2 µl of 10 x buffer, 0.2 µl of Sigma Taq polymerase (5 U/µl), 100 ng of DNA, a final concentration of 200 µM for each dNTP, final concentration of 2.5 mM of $MgCl_2$ and distilled water. The amplification program comprised of an initial denaturation cycle at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C/1 min, annealing at 53°C for CCA primer or 47°C for TGT primer/1 min, extension at 72°C/2 min, and an additional cycle including a 10-min extension at 72°C. The cycles were carried out in a Hybaid Express Thermal Cycler. The 20 µl of the PCR products were electrophoresed in 1.5% SeaKem LE agarose at 100V for 3.5 h, after which the gel was stained in ethidium bromide (0.5 µg/ml) and photographed. A Gibco low DNA mass ladder was used as a marker. Banding patterns were analysed by unweighted pair group method (UPGMA) band matching using GelCompar II software (Applied Maths, Kortrijk, Belgium).

For AFLP the genomic DNA was digested both with a regular-cutting (*Hpa*II) and a rare-cutting (*Eco*RI) restriction enzyme and adapters were ligated to the resultant fragments using a method modified from Vos *et al*. (1995). In this study, AFLP banding patterns were generated with three separate primer pairs GC-ACC, AT-CTC, and AT-ACC, and analysed by UPGMA band matching using GelCompar II software (Applied Maths, Kortrijk, Belgium) (Aquino de Muro *et al*. 2003).

Results and discussion

i) Isolation and identification.

During the search for fungal isolates, CABI Bioscience has processed approximately 70,000 insect cadavers. Many small samples of a few hundred insects were obtained from most sites. However, very large numbers were obtained from a few sites and these cadavers were processed in groups of thousands. One isolate per 10,000 insects is comparable to that found in work with similar pests in Kenya (Oduor *et al*, 2000). Eight isolates obtained from farm stores in England were identified as *Beauveria bassiana*.

ii) Biological characterisation.

For most isolates, the preferred temperatures for growth were 25°C and 30°C (only 386605 and 387294 showed better growth at 25°C), and for sporulation were 20°C and 25°C. The fact that the single spore cultures of a particular multiple spore isolate sporulated better at a range

of different temperatures (e.g. 386368) suggests that multiple spore isolate is adaptable to different temperatures. It would therefore be a better candidate for mass production and field application than another where the single spores showed maximal sporulation at similar temperatures (e.g. 387294). At 15ºC there is less variability in the growth than at other temperatures. There is less growth at this temperature than at higher temperatures greatest variability tends to occur at 30ºC. Isolate 386243 has much higher growth rates than the other isolates at temperatures greater than 15ºC. Isolate 386243 performs similarly at temperatures of 20ºC and 25ºC for both growth and sporulation. Growth is similar at 30ºC but sporulation is lower than at 20ºC and 25ºC. Isolate 387294 behaves relatively similarly at all temperatures, particularly above temperatures of 15ºC.

In general, there are differences in the growth at different temperatures – as temperature increases growth increases. For sporulation there are less distinct differences at each temperature. For isolates 386243 and 386606 sporulation at temperatures of 15ºC and 30ºC are similar and lower than sporulation at temperatures of 20ºC or 25ºC.

iii) Molecular characterisation.

The ITS analyses did not show any intraspecific variation amongst the isolates. (The full sequencing results for the molecular characterisation of isolates are available in Appendix A.) On the other hand, the results from ISSR-PCR and AFLP analyses supported each other. Figure 1 shows the results for AFLP cluster analysis using Band Matching and UPGMA - combined dendrogram of relatedness (Dice similarity coefficient) prepared from AFLP banding patterns for all 19 isolates, including original multiple spore, and respective re-isolated multiple spore and single spore cultures. The AFLP dendrogram shows 5 main clusters, and grouping of IMI 386605, IMI 386606, IMI 386607 and IMI 386367 isolates in one cluster, but isolate IMI 386243, although isolated from the same farm in Leicestershire was clustered separately. The isolate IMI 387294 was also clustered separately. These results showed genetic diversity amongst the *B. bassiana* isolates from UK farms, revealing particularly the presence of more than one isolate within the same UK farm premises.

Figure 1. Cluster analysis using Band Matching and UPGMA. Combined dendrogram of relatedness (Dice similarity coefficient) prepared from AFLP banding patterns for all 19 isolates, including original multiple spore, and respective re-isolated multiple spore and single spore cultures. IMI 386243, IMI 386605, IMI 386606, IMI 386607, and IMI 386367 were isolated from a farm in Leicestershire; IMI 386368 was isolated from a farm in Bedfordshire; IMI 387294 was isolated from a farm in Suffolk.

AFLP (AT-CTC+AT-ACC+GC-ACC)

% relative similarity

B. bassiana IMI 387294 R D	England
B. bassiana IMI 387294 R C	England
B. bassiana IMI 387294 R E	England
B. bassiana IMI 387294 R A	England
B. bassiana IMI 387294 M	England
B. bassiana IMI 387294 R B	England
B. bassiana IMI 387294 R	England
B. bassiana IMI 382231 M	Turkey
B. bassiana IMI 382231 R	Turkey
B. bassiana IMI 382297 M	Turkey
B. bassiana IMI 382297 R	Turkey
B. bassiana IMI 382239 M	N. Afghanistan
B. bassiana IMI 382239 R	N. Afghanistan
B. bassiana IMI 173201 R	UK
B. bassiana IMI 173199 M	UK
B. bassiana IMI 173199 R	UK
B. bassiana IMI 173201 M	UK
B. bassiana IMI 187643 M	Adriatic sea
B. bassiana IMI 187643 R	Adriatic sea
B. bassiana IMI 061345 R	UK
B. bassiana IMI 386243 R D	England
B. bassiana IMI 386243 R E	England
B. bassiana IMI 386243 M	England
B. bassiana IMI 386243 R C	England
B. bassiana IMI 386243 R B	England
B. bassiana IMI 386243 R	England
B. bassiana IMI 386243 R A	England
B. bassiana IMI 386607 R	England
B. bassiana IMI 386607 R B	England
B. bassiana IMI 386607 M	England
B. bassiana IMI 386607 R A	England
B. bassiana IMI 386367 R E	England
B. bassiana IMI 386607 R D	England
B. bassiana IMI 386607 R C	England
B. bassiana IMI 386607 R E	England
B. bassiana IMI 386605 R A	England
B. bassiana IMI 386605 R C	England
B. bassiana IMI 386605 R B	England
B. bassiana IMI 386605 R D	England
B. bassiana IMI 386367 R C	England
B. bassiana IMI 386367 R B	England
B. bassiana IMI 386367 R D	England
B. bassiana IMI 386606 R E	England
B. bassiana IMI 386367 M	England
B. bassiana IMI 386367 R A	England
B. bassiana IMI 386606 R C	England
B. bassiana IMI 386606 R B	England
B. bassiana IMI 386606 R A	England
B. bassiana IMI 386605 M	England
B. bassiana IMI 386367 R	England
B. bassiana IMI 386606 M	England
B. bassiana IMI 386606 R	England
B. bassiana IMI 386606 R D	England
B. bassiana IMI 386605 R	England
B. bassiana IMI 386605 R E	England
B. bassiana IMI 386242 M	Iran
B. bassiana IMI 358840 R	England UK
B. bassiana IMI 358840 M	England UK
B. bassiana IMI 061345 M	UK
B. bassiana IMI 386368 M	England
B. brongniartii IMI 303228 M	UK
B. brongniartii IMI 303228 R	UK
B. brongniartii IMI 223216 M	UK
B. brongniartii IMI 223216 R	UK
Sporothrix sp. IMI 385519 M	England

MILESTONE 3a. Quantify and compare the activity of these fungi, together with isolates already in culture at CABI Bioscience, against storage invertebrates under environmental conditions that are appropriate to UK grain stores.

Methods and materials

Primary screening has been undertaken of eleven *Beauveria bassiana* and two *Beauveria brongniartii* fungal isolates, prepared by CABI Bioscience from their existing culture collection and from the recently collected field samples (Tables 1-4). Ten UK isolates and, for comparative purposes, four non-UK isolates were selected for maximum challenge efficacy tests against four pest species, chosen to represent the different types of invertebrate pests commonly found in UK mills and stores: the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Tram 9213), the Mediterranean flour moth, *Ephestia kuehniella* (lab), the black domestic psocid, *Lepinotus patruelis* (GB), and the flour mite, *Acarus siro* (9258/2).

For the insect species, four replicates each of either 15 beetle adults, 15 psocid adults or 15 moth larvae were used for each treatment, together with 4 control replicates. For each treatment, insects were tipped carefully on to the fungal isolate growing on an agar plate. Individual insects were gently rolled over using soft forceps or a fine paint brush to ensure fungal conidia adhered to all parts of the insect exoskeleton. Control insects were treated in the same way but on plates containing agar without fungus. The insects were then transferred to 9 cm diameter Petri dishes containing filter papers moistened with 750 µl sterile distilled water, sealed with 'Parafilm' and kept at 20°C, 70%RH. After 24 hours the insects were transferred to 120 ml wide necked glass jars containing approx. a 10 mm depth of their culture food (wheatfeed and dried yeast powder mixed with either rolled oats for beetles, glycerol for moths, or skimmed milk powder and wholemeal flour for psocids).

Mortality was assessed every few days for up to 10 days after treatment or until all the insects had died if shorter. Dead insects were removed at each 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%RH they were examined for external sporulation of fungus to confirm that death was most likely due to mycosis caused by the fungal isolate.

To reduce the amount of handling required for the smaller and more delicate mites, freeze-dried fungal spores were mixed with 0.5 ml of sterile distilled water and 50 µl of the suspension was applied to a 4.25 cm diameter Whatman No. 1 filter paper previously moulded in a metal 'former' to produce a shallow depression measuring 33 mm diameter and 2 mm high as described in Thind and Muggleton (1998). After drying, the treated filter papers were used to prepare mite test cells as described for recovery cells in Thind and Muggleton (1998). A small amount (approx. 3 mg) of mite culture food (wheatgerm and dried yeast powder) was placed in each cell, together with 20 mites. Five replicates were prepared for each treatment, together with five untreated control replicates. The two halves of each cell were clipped together with bulldog clips, and left in a desiccator over water at 20°C. Mortality was assessed every few days for 10 days after treatment or until all the mites were dead. Dead mites were not removed from the cell but damp filter paper was added and the mites checked for mycosis.

Results and discussion

The mean cumulative percentage mortality at 7 and 10 days with each of the fungal isolates for *O. surinamensis*, *E. kuehniella*, *L. patruelis* and *A. siro* are shown in Tables 1-4, respectively.

Table 1. *O. surinamensis* Mean cumulative percentage mortality ± SE at day 7 and day 10. (N=4) (Control response in parentheses)

Isolate reference number	Country of origin	Cumulative mean % mortality ± SE Day 7	Cumulative mean % mortality ± SE Day 10
Beauveria bassiana			
061345	UK	0 ± 0 (1.7 ± 1.7)	0 ± 0 (1.7 ± 1.7)
173199	UK	75.0 ± 9.6 (3.0 ± 3.0)	77.0 ± 8.8 (3.0 ± 3.0)
173201	UK	100 ± 0 (1.7 ± 1.7)	100 ± 0 (1.7 ± 1.7)
358840	UK	87.0 ± 2.7 (3.0 ± 3.0)	88.5 ± 1.5 (3.0 ± 3.0)
386243	UK	81.3 ± 5.0 (1.7 ± 1.7)	91.4 ± 1.9 (3.3 ± 3.3)
386368	UK	85.4 ± 10.1 (1.8 ± 1.8)	85.4 ± 10.1 (1.8 ± 1.8)
387294	UK	35.0 ± 11.0 (2.0 ± 2.0)	39.0 ± 13.0 (3.0 ± 3.0)
389521	UK	100 ± 0 (1.8 ± 1.8)	100 ± 0 (3.5 ± 2.0)
187643	Adriatic	90.0 ± 4.3 (0)	93.0± 2.7 (0)
382297	Turkey	79.0 ± 7.8 (0)	84.0 ± 7.5 (0)
382239	N. Afghanistan	58.5 ± 7.8 (0)	75.0 ± 3.0 (0)
382231	Turkey	91.5 ± 3.2 (0)	93 ± 2.7 (0)
B. brongniartii			
223216	UK	5.0 ± 1.7 (1.7 ± 1.7)	5.0 ± 1.7 (1.7 ± 1.7)
303228	UK	5.5 ± 1.8 (10.0 ± 4.3)	5.5 ± 1.8 (10.0 ± 4.3)

Table 2. *E. kuehniella* Mean cumulative percentage mortality ± SE at day 7 and day 10. (N=4)
(Control response in parentheses)

Isolate reference number	Country of origin	Cumulative mean % mortality ± SE Day 7	Cumulative mean % mortality ± SE Day 10
Beauveria bassiana			
061345	UK	16.2 ± 9.3 (5.5 ± 3.5)	25.0 ± 6.8 (10.9 ± 4.7)
173199	UK	68.0 ± 10.9 (8.5 ± 4.2)	97.0 ± 3.2 (18.5 ± 6.4)
173201	UK	100 ± 0 (5.5 ± 3.5)	100 ± 0 (10.9 ± 4.7)
358840	UK	91.5 ± 6.4 (8.5 ± 4.2)	100 ± 0 18.5 ± 6.4)
386243	UK	72.4 ± 9.6 (1.8 ± 1.8)	84.8 ± 11.0 (10.7 ± 4.6)
386368	UK	94.7 ± 1.7 (8.8 ± 1.5)	100 ± 0 (12.4 ± 3.3)
387294	UK	50.0 ± 7.0 (7.0 ± 3.0)	59.0 ± 6.0 (9.0 ± 4.0)
389521	UK	100 ± 0 (5.2 ± 3.4)	100 ± 0 (7.0 ± 5.1)
187643	Adriatic	97.0 ± 3.2 (2.0 ± 2.0)	98.0 ± 1.7 (7.0 ± 2.9)
382297	Turkey	96.5 ± 3.5 (2.0 ± 2.0)	96.5 ± 3.5 (7.0 ± 2.9)
382239	N. Afghanistan	87.0 ± 8.7 (2.0 ± 2.0)	94.5 ± 3.6 (7.0 ± 2.9)
382231	Turkey	83.0 ± 5.6 (2.0 ± 2.0)	95.0 ± 5.0 (7.0 ± 2.9)
B. brongniartii			
223216	UK	10.2 ± 4.4 (5.5 ± 3.5)	10.2 ± 4.4 (10.9 ± 4.4)
303228	UK	7.0 ± 4.7 (3.0 ± 3.0)	10.5 ± 6.5 (8.5 ± 5.1)

Table 3. *L. patruelis* Mean cumulative percentage mortality ± SE at day 7 and day 10. (N=4) (Control response in parentheses)

Isolate reference number	Country of origin	Cumulative mean % mortality ± SE Day 7	Cumulative mean % mortality ± SE Day 10
Beauveria bassiana			
061345	UK	61.1 ± 6.4 (30.7 ± 10.4)	63.2 ± 8.0 (34.0 ± 8.9)
173199	UK	100 ± 0 (20.0 ± 10.6)	100 ± 0 (25.0 ± 7.0)
173201	UK	100 ± 0 (30.7 ± 10.4)	100 ± 0 (34.0 ± 8.9)
358840	UK	100 ± 0 (20.0 ± 10.6)	100 ± 0 (25.0 ± 7.0)
386243	UK	100 ± 0 (29.1 ± 4.9)	100 ± 0 (30.8 ± 4.1)
386368	UK	98.2 ± 1.8 (14.4 ± 5.7)	98.2 ± 1.8 (16.0 ± 5.2)
387294	UK	56.3 ± 12.8 (26.1 ± 6.7)	61.9 ± 12.8 (32.0 ± 10.3)
389521	UK	100 ± 0 (15.5 ± 1.6)	100 ± 0 (17.4 ± 2.5)
187643	Adriatic	100 ± 0* (32.0 ± 14.5)	100 ± 0 (36.0 ± 13.5)
382297	Turkey	100 ± 0* (32.0 ± 14.5)	100 ± 0 (36.0 ± 13.5)
382239	N. Afghanistan	100 ± 0* (32.0 ± 14.5)	100 ± 0 (36.0 ± 13.5)
382231	Turkey	100 ± 0* (32.0 ± 14.5)	100 ± 0 (36.0 ± 13.5)
B. brongniartii			
223216	UK	36.6 ± 4.8 (30.7 ± 10.4)	38.4 ± 5.0 (34.0 ± 8.9)
303228	UK	38.8 ± 10.3* (44 ± 4.0)	44 ± 9.8 (49 ± 2.1)

* Mean cumulative percentage mortality ± SE at day 6

Table 4. *A. siro.* Mean cumulative percentage mortality ± SE at day 7 and day 10. (N=5) (Control response in parentheses)

Isolate reference number	Country of origin	Cumulative mean % mortality ± SE Day 7	Cumulative mean % mortality ± SE Day 10
Beauveria bassiana			
061345	UK	15.0 ± 7.1 (6.0 ± 1.9)	44.0 ± 10.7 (9.0 ± 2.9)
173199	UK	5.0 ± 2.7 (5.0 ± 2.2)	33.0 ± 3.7 (6.0 ± 2.9)
173201	UK	50.0 ± 7.9 (6.0 ± 1.9)	100 ± 0 (9.0 ± 2.9)
358840	UK	1.0 ± 1.0 (5.0 ± 2.2)	18.0 ± 4.1 (6.0 ± 2.9)
386367	UK	95.0 ± 3.9 (3.0 ± 2.0)	100 ± 0 (5.0 ± 1.6)
386368	UK	99.0 ± 1 (3.0 ± 2.0)	100 ± 0 (5.0 ± 1.6)
387294	UK	7.0 ± 2.0 (1.0 ± 1.0)	38.0 ± 2.0 (3.0 ± 3.0)
187643	Adriatic	15.0 ± 5.2 (5.0 ± 2.2)	85.0 ± 7.6 (6.0 ± 2.9)
382297	Turkey	8.0 ± 4.6 (5.0 ± 2.7)	13.0 ± 4.4 (8.0 ± 4.1)
382239	N. Afghanistan	11.0 ± 3.7 (5.0 ± 2.7)	39.0 ± 10.7 (8.0 ± 4.1)
382231	Turkey	24.0 ± 7.9 (5.0 ± 2.7)	49.0 ± 11.3 (8.0 ± 4.1)
B. brongniartii			
223216	UK	13.0 ± 3.0 (6.0 ± 1.9)	24.0 ± 4.8 (9.0 ± 2.9)
303228	UK	21.0 ± 4.3 (5.0 ± 2.7)	28.0 ± 6.4 (8.0 ± 4.1)

For the beetles, moths and mites, control mortalities were usually less than 10%. For the psocids, control mortalities were higher due to their more delicate bodies. No fungal growth was observed on dead insects or mites from control treatments. The majority of beetles, psocids, mites and moth larvae from treated plates showed signs of fungal growth five days after surface disinfection. In some cases this growth was visible within 48 hours.

The 'high challenge' test used for the primary screen provided a quick method of determining which isolates were pathogenic to the pest species. However, the pest species were exposed to unnaturally high levels of spores; we also needed to know how pathogenic these isolates were at lower spore concentrations, and this requirement was addressed through a secondary screen.

From the results of the primary screen, it was decided that UK isolates 061345, 223216 and 303228 should not be taken forward to the secondary screen because of the low mortality observed compared to other isolates. The remaining UK isolates were taken forward to the secondary screen. Non-UK isolates were excluded from the secondary screen because of possible quarantine implications associated with their use in UK stores and mills.

MILESTONE 3b. Conduct further screening of isolates.

Methods and materials

Additional studies were conducted to find and validate a bioassay method, which gave good differentiation between the isolates whilst maintaining conditions similar to those found in UK stores. Secondary screen dose response tests were then conducted on the seven most promising isolates from the primary screen.

A Potter Tower was used to spray 1ml of spores suspended in a carrier of a 1:4 mix of Codacide and sterile distilled water. Based on an assessment at CABI Bioscience of the number of viable conidia per gm of material, four log doses in the concentration range 1×10^5 – 1×10^8 conidia/ml were prepared by serial dilution and tested against all four representative pest species. Petri dishes containing filter papers (90 mm diameter) were sprayed with the spore suspension for tests with the psocid and moth larvae and the filter papers were allowed to dry before insects were confined to the treated surface for 24 hours. For tests with the beetles, the method was adapted and the insects were placed in the Petri dishes prior to spraying of the spore suspension so that the beetles were sprayed directly. A filter paper dampened with sterile distilled water was placed in the lid of the Petri dishes for the first 24 hour period. After 24 hours insects were transferred to clean untreated filter papers in Petri dishes with a little food and kept in a controlled environment room at 20ºC 70%RH for 10 days.

For the mites, 42.5mm diameter sprayed filter papers were used to prepare standard CSL mite recovery cells (Thind and Muggleton, 1998) in which the mites were confined for 24 hours, in a desiccator containing water. After 24 hours, mites were transferred to clean untreated cells and kept at 20ºC in a desiccator providing 80%RH. Mortality was assessed after 7 and 10 days.

Twenty insects or mites were used in each replicate, and there were five replicates at each concentration. Controls were treated with carrier only. As in the primary screen, dead insects and mites were retained to confirm that death was due to mycosis.

We looked at the effects of a few different temperature and RH combinations on the efficacy of four of the best isolates at the highest dose of $1x10^8$ conidia/ml against the beetle *Oryzaephilus* and the mite *Acarus.* These tests were conducted at 15°C 80%RH and 20°C 80%RH <u>without</u> the extra period of high RH for the first 24 hours but <u>with</u> the spraying of beetles directly.

Dose response tests incorporating the period of high RH for the first 24 hours were also completed using the four top isolates against some additional insect and mite species: the grain weevil *Sitophilus granarius* (Gainsborough), the rust red grain beetle *Cryptolestes ferrugineus* (Stow), the confused flour beetle *Tribolium confusum* (W44), the grainstack mite *Tyrophagus longior* (T101), and an OP susceptible strain of the saw toothed grain beetle *Oryzaephilus surinamensis*.
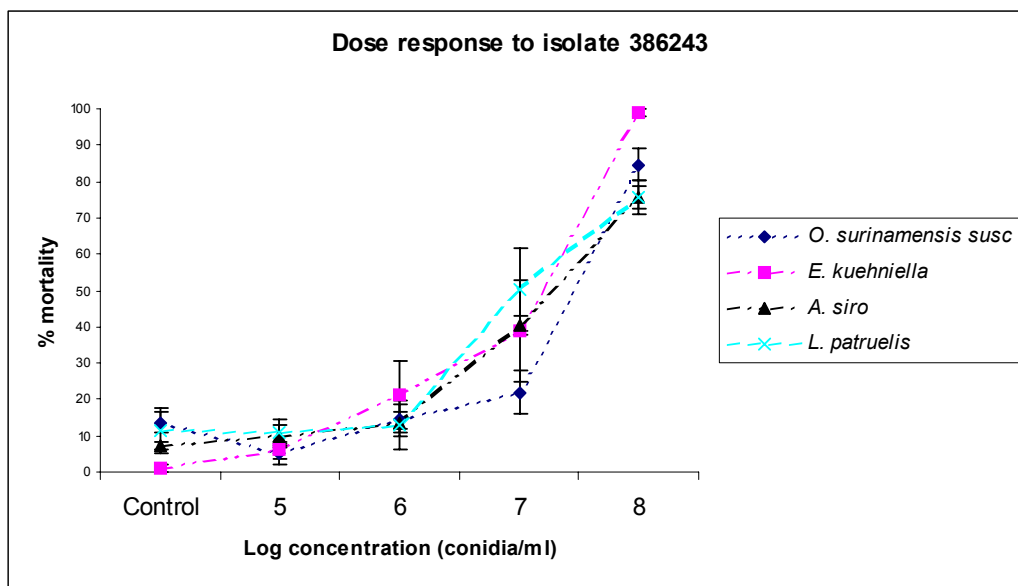
Results and discussion

The 1:4 mix of Codacide and water was chosen because oil carriers are considered to be particularly effective in dry conditions, readily wetting the hydrophobic, lipophilic surfaces of insects, and acting as penetrants and stickers. Codacide is an oilseed rape product containing a small amount of emulsifier, with the added advantage to Organic Farmers that it is a naturally derived plant product rather than an industrial mineral oil. Also, when this type of material is sprayed, the fine droplets should carry well into hidden dead spaces in the fabric of a store. Water was added to reduce viscosity and prevent the Codacide clogging up the spray nozzle in the Potter Tower. The choice of concentrations was based on those shown to be effective against other stored food pests (Hluchy and Samsinakova, 1989; Adane *et al*., 1996; Smith *et al.,* 1998).

In the secondary screen, the highest mortalities in all four pest species occurred at the maximum dose of 1×10^8 conidia/ml with all the isolates tested. Isolate 386243 gave the best results for both insects and mites; mortality was close to 100% for *O.* surinamensis and *E. kuehniella,* and around 75% for *A. siro* and *L. patruelis* (Table 5). Mortalities for controls treated with carrier were usually below 10%.

Table 5. Mean percentage mortality ±SE at 1×10^8 conidia/ml 10 days after exposure to the top four isolates against nine pest species/ strains. (N=5) (Control response in parentheses)

	386243	386606	386367	389521
Oryzaephilus surinamensis Tram	95.7 ± 2.6 (11.4 ± 4.8)	86.1 ± 10.3 (11.4 ± 4.8)	42.1 ± 11.5 (2.0 ± 1.2)	63.0 ± 10.7 (3.5 ± 2.5)
Ephestia kuehniella	99.0 ± 1.0 (1.05 ± 1.05)	87.3 ± 6.3 (1.05 ± 1.05)	95.9 ± 2.9 (8.6 ± 1.5)	86.2 ± 5.3 (14.0 ± 2.4)
Lepinotus patruelis	75.6 ± 4.5 (11.5 ± 6.2)	26.5 ± 5.4 (5.3 ± 3.4)	38.3 ± 4.4 (5.3 ± 2.3)	69.3 ± 8.5 (6.4 ± 5.1)
Oryzaephilus surinamensis susc	84.7 ± 4.5 (13.6 ± 2.9)	47.0 ± 8.7 (2.0 ± 2.0)	11.7 ± 3.4 (7.7 ± 6.5)	83.0 ± 5.6 (3.3 ± 2.3)
C. ferrugineus	89.0 ± 12.3 (6.94 ± 6.94)	86.0 ± 8.3 (10.1 ± 5.47)	100 ± 0 (6.94 ± 6.94)	100 ± 0 (3.0 ± 2.0)
S. granarius	65.0 ± 5.7 (1.0 ± 1.0)	27.5 ± 4.0 (1.0 ± 1.0)	1.0 ± 1.0 (3.95 ± 3.95)	3.0 ± 1.2 (0 ± 0)
T. confusum	27.0 ± 11.0 (38.4 ± 8.5)	6.3 ± 3.9 (6.0 ± 2.4)	1.0 ± 1.0 (2.0 ± 1.2)	1.0 ± 1.0 (0 ± 0)
A. siro	75.6 ± 3.0 (7.2 ± 1.3)	48.5 ± 5.4 (13.0 ± 2.0)	46.2 ± 4.3 (5.7 ± 2.7)	20.0 ± 7.7 (5.6 ± 2.9)
T. longior	9.1 ± 1.4 (5.0 ± 2.2)	4.7 ± 3.1 (2.0 ± 1.2)	8.7 ± 2.6 (2.0 ± 1.2)	10.1 ± 2.4 (1.0 ± 1.0)

The full range of dose response figures for the beetle, *Oryzaephilus surinamensis,* the mite, *Acarus siro,* the psocid, *Lepinotus patruelis,* and larvae of the moth, *Ephestia kuehniella* using isolate 386243 are shown in Figure 2, with the highest mortalities occurring at the maximum dose of 1×10^8.

Figure 2. Dose response of 3 insect and 1 mite species to fungal isolate 386243

In the tests conducted at 15°C 80%RH and 20°C 80%RH without the extra period of high RH for the first 24 hours but with the spraying of beetles directly, 386243 was again the best isolate for *O. surinamensis* and *A. siro*. As expected, efficacy was significantly reduced at the lower temperature: mortality for the mites at 80%RH fell from 52% at 20°C to 34% at 15°C. For *O. surinamensis* mortality at 80%RH fell from 9% at 20°C to 0% at 15°C. Standard errors ranged from +/- 6.5 to 2.4. Generally, there was little improvement in efficacy when the RH was raised from 70% to 80%. When the extra 24 hour humidification period was included in the treatment, mortality for *O. surinamensis* and isolate 386243 at 15°C 80%RH rose to 42% with a standard error of +/-5.1.

To ensure high mortality and good differentiation between isolates, it was necessary to keep insects, particularly the beetles, and to a lesser extent mites, on the treated papers in a humid atmosphere close to 100%RH for the first 24 hours before transferring the insects to 70%RH and the mites to 80%RH for the remaining 9 days. The reason for this requirement for high RH is not clear but it may have important implications for the way any future product is formulated and used in grain stores. We also found that to obtain high mortality for the beetles, it was necessary to spray them directly rather than just allowing them to crawl over sprayed substrates which was sufficient for the other insects and mites. We have spent some

time investigating these findings and they will be discussed in more detail when formulation issues are considered under Milestone 6.

The results for the four best isolates at the highest dose against the five additional pest species and strain are also presented in Table 5. Again isolate 386243 gave the highest mortalities overall. All four isolates were highly effective against the rust red grain beetle but were much less effective against the confused flour beetle or the grainstack mite.

These results emphasise the poor efficacy obtained without the extra 24-hour humidification period, particularly for the beetles. They also give some indication of the likely reduction in efficacy as temperatures in grain stores fall. However, higher pest mortalities are likely at the lower temperatures after longer exposure periods. Also, in future it may be possible to select isolates that are more effective at the lower temperatures.

MILESTONES 4 and 5. Compare mycopesticidal efficacy of top candidates with appropriate insecticidal treatments, apply the most promising isolates to pilot-scale storage structures and define their effects on storage pests under practical conditions of temperature and humidity.

(a) Methodologies have been evaluated for assessing efficacy and persistence of formulated fungal isolates applied to different types of grain store building materials:

Methods and materials

The method chosen was similar to that developed for an earlier HGCA project at CSL on the efficacy of pesticides for grain store structure treatments (Cox *et al.,* 2000). Standard samples (30cm squares) of plywood, steel and concrete, together with filter papers for comparison with earlier tests, were precision sprayed with fungal spores using a laboratory sprayer (nozzle tip 9501E) developed at CSL to give uniform deposits on these types of substrates. Isolate 389521 in the 1:4 Codacide: water carrier was applied at a rate of 1.38×10^9 spores/m^2, equivalent to the rate applied in the earlier tests using the Potter Tower sprayer. The sprayed substrates were allowed to dry for 2 hours before insects were added to each, confined to the substrate surface using a metal ring coated with fluon and kept at 20ºC 80%RH. Adults of *O. surinamensis* (Tram) and *L. patruelis*, and late instar larvae of *E. kuehniella* were tested, using 5 replicates of 20 insects for each species and substrate type. After 24 hours, the insects were

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removed from the treated surfaces and transferred to untreated filter papers. Mortality was assessed after 4, 7 and 11days.

Results and discussion

Preliminary tests with the 1:4 Codacide and water mix revealed that this carrier, although acceptable when sprayed onto filter paper for the laboratory dose-response tests, remained very sticky on non-porous surfaces such as steel resulting in insects becoming trapped in it even after several weeks of drying. These and other formulation issues need to be resolved before efficacy testing is resumed, using the most promising fungal isolates against three insect and one mite species. A comparison with the conventional pesticide spray 'Actellic' will also be included, which is one of only two pesticides still registered for use on structures with which grain could come into contact. Persistence will be measured at 1 and 7 days, and 1 and 3 months after spraying.

(b) CABI Bioscience have been investigating the survival and persistence of individual fungal spores on typical grain store construction materials at a range of temperatures and humidities:

Methods and materials

Conidia (spores) of the *B. bassiana* isolate 386243 were used at the University of Reading to develop a predictive equation for spore persistency on galvanised steel plates similar to the materials used in grain storage structures. 'Persistency' is used here to describe how long conidia remain viable after application. 'Longevity' is used to describe how long stored (packaged) conidia remain viable before application.

Dishes containing conidia powder and steel plates treated with conidia were placed in desiccators above saturated salt solutions to assess persistency in nine different temperature/relative humidity storage environments. Persistency was determined by germination tests. The prediction of conidial survival of entomopathogenic fungi is now possible using equations driven by either conidial moisture content or equilibrium relative humidity and temperature (Hong *et al*., 1998, 2001). For *B. bassiana*, the parameters of the viability equation have been estimated (Hong *et al*., 2001). The objectives of this investigation were (i) to test whether *B. bassiana* isolate IMI 386243 behaves similarly to other isolates investigated previously (Hong *et al*., 2001) and (ii) to model the survival of conidia sprayed on a plate surface with storage relative humidity and temperature. The process involves "accelerated" storage processes whereby conidia are held at high

temperatures, in excess of these expected in the field. This information is used to estimate the rates of viability loss at lower temperatures.

In study one, forty five galvanized steel plates (5x5 cm) each containing about $1x10^9$ conidia on one surface, and one dish containing 2 g conidia powder were placed in desiccators above saturated salt solutions maintained at different constant temperatures providing nine different storage environments in the range 10-40ºC and 30-90%RH. Three plates (as 3 replicates) and a small quantity of dry spores from the dish were removed from storage at intervals ranging from one to 28 days, depending upon the environment, for viability testing. At the end of the storage treatment, conidia powder was withdrawn for the determination of equilibrium relative humidity (ERH) and moisture content (MC). ERH was estimated using water activity meter Aqualab CX2 at 20ºC. Moisture content (%, w.b.) was determined at 103ºC for 17 hours. Conidial survival curves were fitted to the observations by probit analysis using GENSTAT 5.

In study two, dry spore powder of *B. bassiana* IMI 386243 was equilibrated at 75 and 33% RH (giving MC 21.8 and 7.8% w.b. respectively). The spores were formulated in Shellsol T and sprayed using an air propellant sprayer onto galvanised steel plates (5x5 cm), at a concentration of $1x10^9$ spores / plate. The plates were then hermetically sealed in individual aluminium foil packets (6.5x9.5 cm). Spraying took place at 30-35% RH for both treatments, but sealing took place after 10 minutes after transferring to CT rooms (20ºC) with RH adjusted to 65-80% and 30-35% according to spore RH. Small samples of dry spore powder were also stored in aluminium foil packets (1x2 cm), and then all treatments were put at 40°C. Three plates and 3 small packets of dry powder were removed at intervals ranging from 1d to 10 d (sampling regime depended on the environment) and assessed for viability. Spores were re-suspended from the steel surfaces and smeared onto SDA plates, incubated at 25°C for 24 hours, and then counts of germinated and non-germinated spores were made.

Results and discussion

In many situations an extended period of viability under field conditions results in greater levels of pest control. Sometimes persistency may be more influential than absolute virulence between isolates. The present work aimed to examine broad effects of different formulations in extending persistency.

The definitions of persistency and longevity are for convenience, to differentiate between different circumstances. However, as far as is known, the biological processes are very

similar; viability is dependent on temperature and moisture content of the conidia. Although this may be more consistent with conidia being stored than those that have been applied and are subject to diurnal variations, the basic principles appear to hold true. The conidium is the resting spore type, which survives for longer periods if its metabolism is slowed as, for example, when it is dry or cool. Germination is an active process requiring water, with increasing speed of germination being associated with greater levels of kill. The present requirement for a period of high humidity to achieve good kill is probably directly related to the effect of this humidity on effective germination.

The results were compared with the survival patterns from longevity studies on 7 other *B. bassiana* isolates (Hong *et al*. 2001). In both studies viability of conidia was lost as RH increased; however, in the current study the viability response to a change in RH (particularly at higher temperatures) was very much smaller.

Study one showed low persistency of this isolate (i.e. low response to storage relative humidity or conidial moisture content). There is a possibility of toxicity of gases released by saturated salt solutions at high storage temperatures. For example, the survival curves of conidia stored over NaBr, K_2CO_3 and $MgCl_2$ are similar despite the large differences in relative humidity and moisture content. We suspect that the gases released by several saturated salt solutions may have direct (e.g. $MgCl_2$ has been used as disinfectant, $NaNO_2$ evolves a toxic fume of N_2O_3) or indirect effect on viability through N_2, CO_2 or O_2.

In an attempt to clarify: i) if the oil formulated spores would influence the way persistency changed under temperature and / or RH fluctuations; or ii) if the various environments controlled by saturated salt compounds could have been harmful in any way, iii) and to set a clear protocol for future work, a series of small trials were performed. One such test, described here as study two, focused on previous speculation that environments controlled by saturated salt compounds could have a negative effect on spore persistency. In an attempt to eliminate the possibility of such effects occurring this test dispensed with all salt compounds, instead controlling RH by hermetically sealing the steel plates inside aluminium packets.
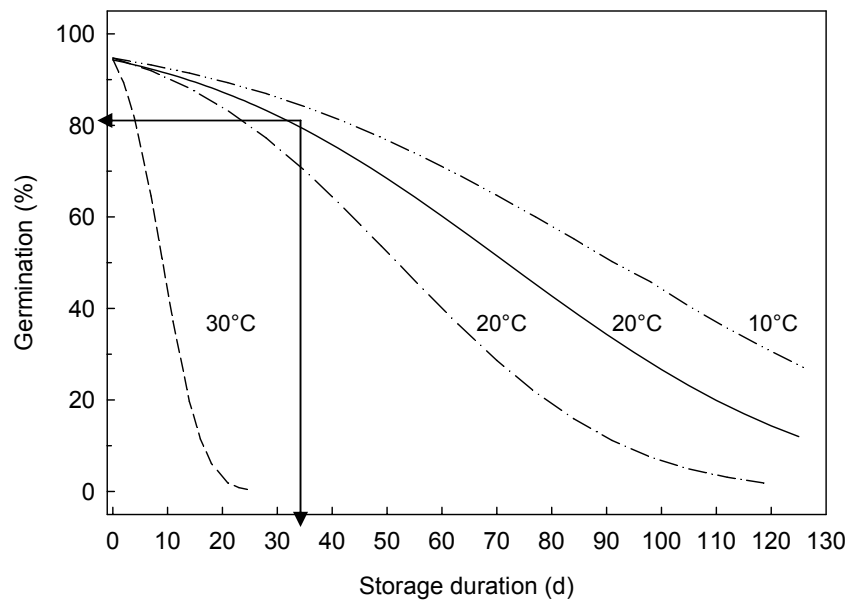
The results of these studies showed that after 35 days at 85%RH and 20°C the conidia in oil formulation have maintained 80% viability (Figure 3), whereas after nearly 80 days under the same conditions the conidia in powder formulation have maintained 80% viability (Figure 4). The broken lines in these figures predict survival of conidia using the parameters of the viability equation for seven isolates of *B. bassiana* (Hong *et al.*, 2001) for 85%RH with 30ºC,

85%RH with 20ºC, and 88%RH with 10ºC. Eighty percent viability is an arbitrary figure for good efficacy of a mycopesticide. In effect, the results are equivalent to the active life of a pesticide; in this case two different formulations result in the mycopesticide being, in theory, capable of persisting for between 5 and 11 weeks, superior to that of many chemicals.
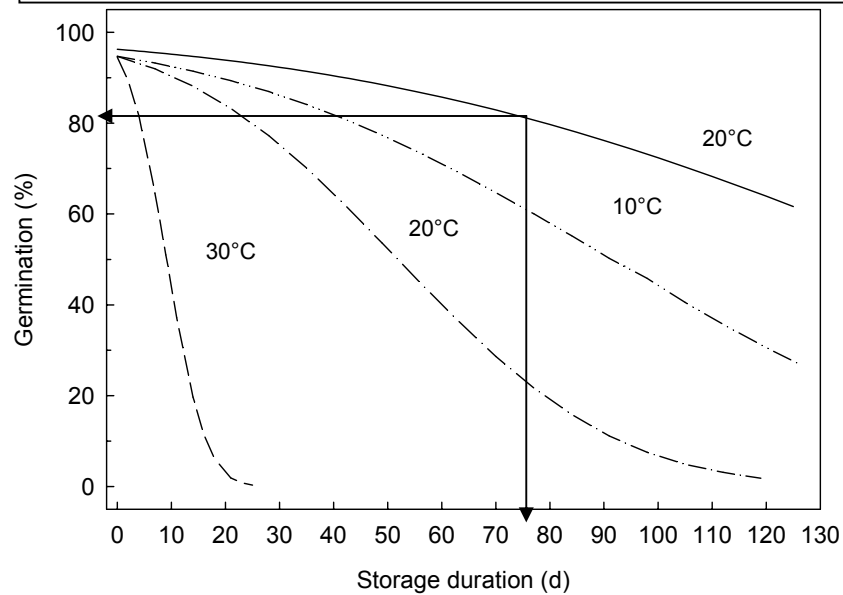
Thus the conidia showed higher persistency in dry powder form than when formulated in oil. Relative humidity appeared to have no effect on the survival of formulated conidia on steel plates. The survival of formulated conidia on steel plates in sealed packets containing an air of 33% or 77%RH was superior to those stored over salt solutions of NaCl (75% RH) or $MgCl_2$ (32%RH). The results suggest that some salts used in the saturated salt solutions technique, may have unknown biological effects, which could cast some doubt on results. Other salts do not have this effect. The survival curves of conidia stored hermetically at 21.8% moisture content (77%RH) or over NaCl (75%RH, 19.5% moisture content) are similar. There was some evidence of toxicity for conidia stored over $MgCl_2$.

Figure 3. Survival curves for oil formulated conidia of isolate 386243 stored at 20ºC 85%RH, fitted by probit analysis to a common origin.

Figure 4. Survival curves for dry powder formulated conidia of isolate 386243 stored at 20ºC 85%RH, fitted by probit analysis to a common origin.

MILESTONE 6. Mass-produce and formulate promising isolates for use in practical-scale trials.

(a) CABI Bioscience completed small-scale mass production of dry spore powder of 7 of the UK *B. bassiana* isolates (low mortality results were obtained for IMI 387294, so this isolate was not included in the mass production line).

Methods and materials
i) Mass production method.

The mass production was based on a two-stage technique which included the preparation of a fungal culture in liquid medium, which was then used to inoculate the solid substrate (rice) (Jenkins *et al*, 1998). The spores were harvested using the CABI MycoHarvester, which was designed to collect particles in an optimum range of approximately 3-10μm (http://www.dropdata.net/mycoharvester/beast.htm). The dry spore powder was packaged in various sizes and a small amount was used for the viability/ contamination tests as part of the quality control of the mass production (Jenkins & Grzywacz, 2000).

ii) Mass Production Quality Control.

To determine the amount of conidia per gram of powder, a pre-weighed amount of conidia powder was placed in a test-tube to which 10 ml of sterile 0.05% Tween 80 (Sigma) was added. Then resulting suspension was vortexed and then sonicated for 10 minutes in a water bath sonicator (Branson 2200, Connecticut, USA) to break up any conidial clumps; the suspension was then further vortexed, diluted and counted in a haemocytometer.

To determine the germination rate of spores, a total of 100-200 mg of conidia powder or a few grains of conidiated substrate were placed on empty 50 mm Petri dishes. The dishes were floated on water in a humid chamber for 30 minutes to permit slow re-hydration of the dry conidia. A suspension of conidia was then prepared by placing rehydrated conidia into 10 ml Shellsol T. The resulting spore suspension was adjusted to approximately 1×10^6 conidia/ml and treated in a bath sonicator for 3 min. A small drop of the sonicated suspension was transferred evenly onto the surface of a 50mm SDA Petri dish. Three replicate dishes were prepared for each sample. The dishes were left in a 25°C incubator for 24 h, and then examined microscopically under x200 magnification. A total of at least 300 conidia was counted for each plate. A germinated spore was defined as a spore having a germ tube. Germination rate was then calculated as the ratio of germinated conidia to the total amount of conidia counted and expressed as percentage.

Moisture content of the spore powder was determined using the HG53 Halogen Moisture Content Analyser. A total of 0.8-1 g of conidia were used for each determination; average of two replicate measurements was used.

To determine biological purity, 8 serial dilutions of the conidia suspensions from different batches were prepared, 0.2 ml from each dilution spread on the surface of 2 SDA plates, and incubated for 6 days. For each dilution two replicate plates were set up. Percentage of contamination was calculated as the ratio of the amount of colony forming units (CFUs) of contaminants to the amount of *M. anisopliae* colonies.

Results and discussion

There was considerable variation of the yields, and the mass production proved challenging for some of the isolates when using our standard protocol (Table 6). Also, it would be interesting to investigate the 'pink' colouration observed for IMI 173199, IMI 173201, IMI 358368, and IMI 358840. These isolates were perfectly fine (white and powdery) on SDA agar plates, but seem to produce some sort of metabolite when in liquid or solid media.

Table 6. Mass production of *B. bassiana* isolates.

Isolates	**Kg** of solid substrate (rice)	**g** of dry spore powder	**Yield** g of powder per Kg rice	**Viability** viable spores/g
IMI 386243	1.5 (3)	54.95	36.62	5×10^9
IMI 386605 R	4	4.50	1.125	3.8×10^{10}
IMI 386606 R	4	41.80	10.45	6.4×10^{10}
IMI 386607 R	4	25.84	6.46	5.0×10^{10}
IMI 386367 R	4	40.50	10.125	3.6×10^{10}
IMI 386368	2 (3)	1.44	0.72	2.5×10^9
	6 pink rice	-	0	-
IMI 358840	6	0	0	-
	3 pink rice	-	0	-
IMI 173199	pink liquid	-	0	-
IMI 173201	3 pink rice	-	0	-
IMI 389521	4	85	21.25	2.5×10^{10}
IMI 386243 (MP1)	1.5	54.95	36.63	5.0×10^9
IMI 386243 (MP2)	13	83	6.38	2.5×10^{10}

In the quality control tests, moisture content of packaged dry powder was below 5%, and contamination levels were estimated at lower than 0.002 % of contaminants per colonies of *B.*

bassiana for all dry spore powder from mass productions. Thus, when packaged, the spores should remain viable for at least 3 years at 5-10°C.

(b) Trials using specially prepared 2 metre square plywood arenas have been completed to validate the methodology for testing isolates under practical conditions in the CSL grain storage facility.

Method and materials

A pressurised sprayer was used to treat the inside walls and floor of each arena with isolate 386243 in the 1:4 Codacide and water carrier, 2 hours after 500 adults of *O. surinamensis* had been added. Fluon bands around the walls ensured that insects remained inside the arenas. Four small refuges containing food were placed in each arena 24 hours after treatment to simulate the presence of dead spaces in a grain store. Four replicate arenas treated with spores and carrier were prepared, together with four arenas treated with carrier only. A further two arenas were left completely untreated. Temperature and humidity conditions were recorded using Tiny Tag data loggers. Mortality was assessed 2 weeks after treatment, and dead insects checked for mycosis.

SEM photomicrographs were also prepared at CSL to aid our understanding of spore positions on sprayed surfaces and uptake by insects. Specimens of sprayed beetles and sections of filter paper were sputter-coated with Ag/Pd using an Emscope 500A sputter-coater for 2 minutes at 15mA and examined immediately using a scanning electron microscope (Philips XL20).

Results and discussion

In preliminary tests many beetles died when they became stuck on the Codacide-treated surfaces, making it difficult to interpret the results. However, checking of subsequently surface-sterilised beetles from the treated arenas revealed signs of mycosis in around half of them, demonstrating that they had received lethal doses of spores. These tests again highlighted the problem of the Codacide making the surface structures very sticky, particularly less absorbent areas such as the finer textured areas of plywood sheets, and at lower temperatures, and the need for an improved formulation.
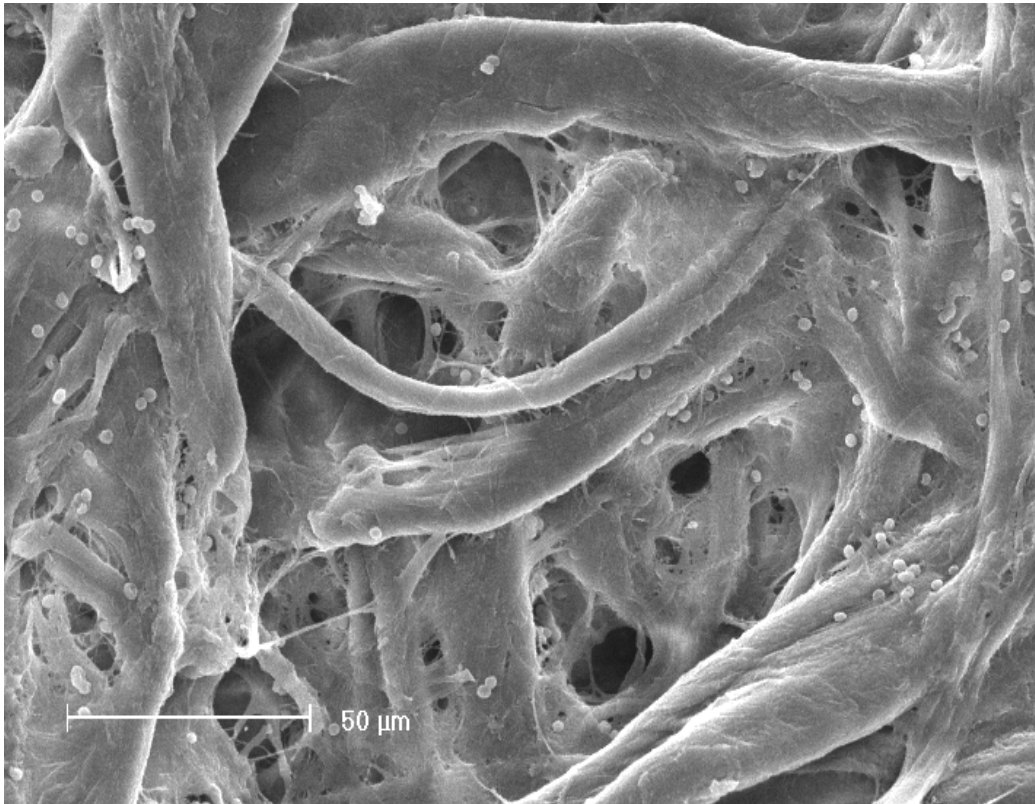
The formulation of test material requires further consideration by formulation specialists. In particular there is a need to address the apparent requirement in the secondary screen for a

short period of high RH when the insects and mites were first placed on treated filter papers to ensure high mortality of all species. Also, the finding that direct spraying of beetles was much more effective than substrate spraying alone has to be considered. The reasons for these requirements are not clear at this stage but they are important in relation to decisions on which formulation and application methods to use in grain stores, so our investigations are continuing.
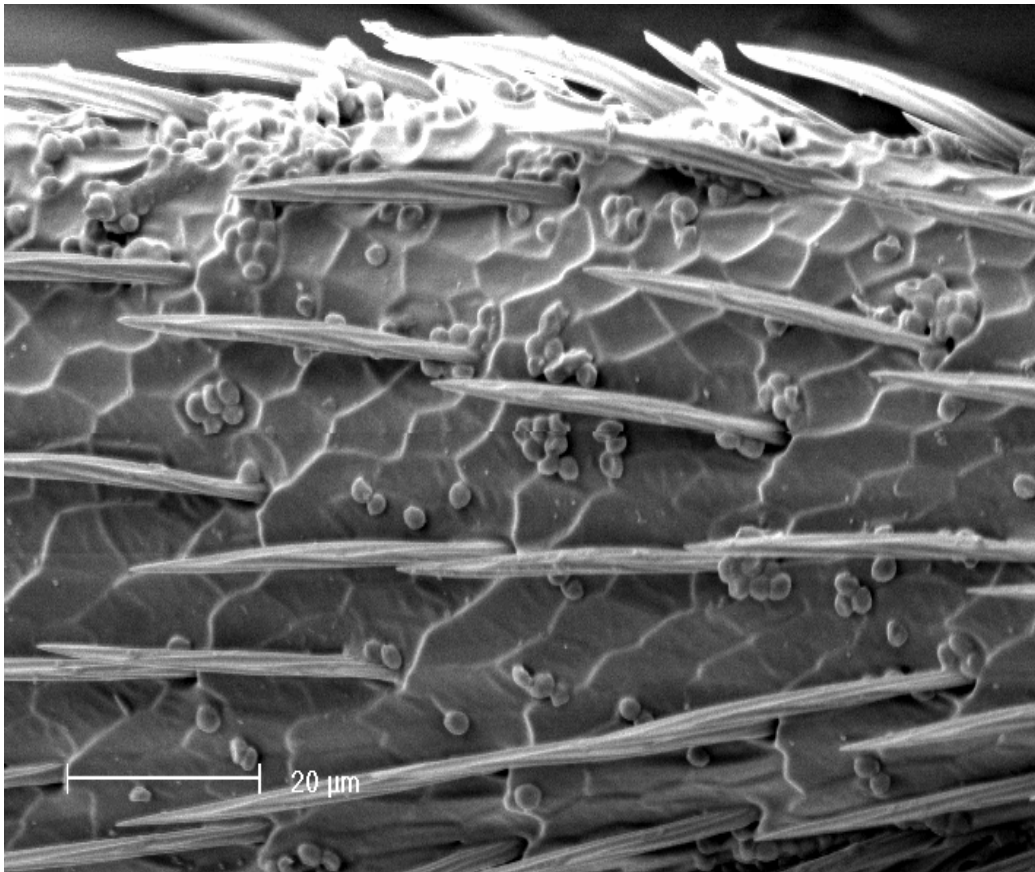
Since the isolates selected for the secondary screen had worked so well in the primary screen where insects were liberally coated with spores, the most likely explanation seems to be that the insects were picking up insufficient spores for mycosis in the Secondary Screen bioassay. The short period of high humidity could be improving spore germination and penetration or enhancing spore uptake. It was also suggested that the fibrous nature of the filter paper used in the secondary screen might have been responsible for the low beetle mortalities when they were not sprayed directly or given extra humidification. As the oily Codacide carrier soaked into the paper and started to dry, it might be binding the fungal spores between the individual fibres and below the surface of the filter paper. In this way fewer of the spores might be accessible to passing insects, thereby reducing the opportunities for spores to adhere to an insect's exoskeleton. A short period of high RH could be slowing down this process and allowing insects to pick up sufficient spores for good control.

SEM photomicrographs were prepared at CSL to aid our understanding of spore positions on filter papers and uptake by insects. Figure 5 is an SEM of a sprayed paper showing spores adhering to individual fibres. Each spore is about 1 micron in diameter. As a beetle walks over this surface, for much of the time only its legs will be in contact with the paper fibres. A moth larva or the smaller psocids and mites are likely to have much more of their body surface in contact with the treated fibres and hence could be picking up more spores. The SEM in Figure 6 reveals plenty of spores attached to part of a leg of *O. surinamensis* after walking over the treated surface, while in Figure 7 fewer spores are found on a section of the ventral surface of the beetle abdomen in the vicinity of some spiracles.
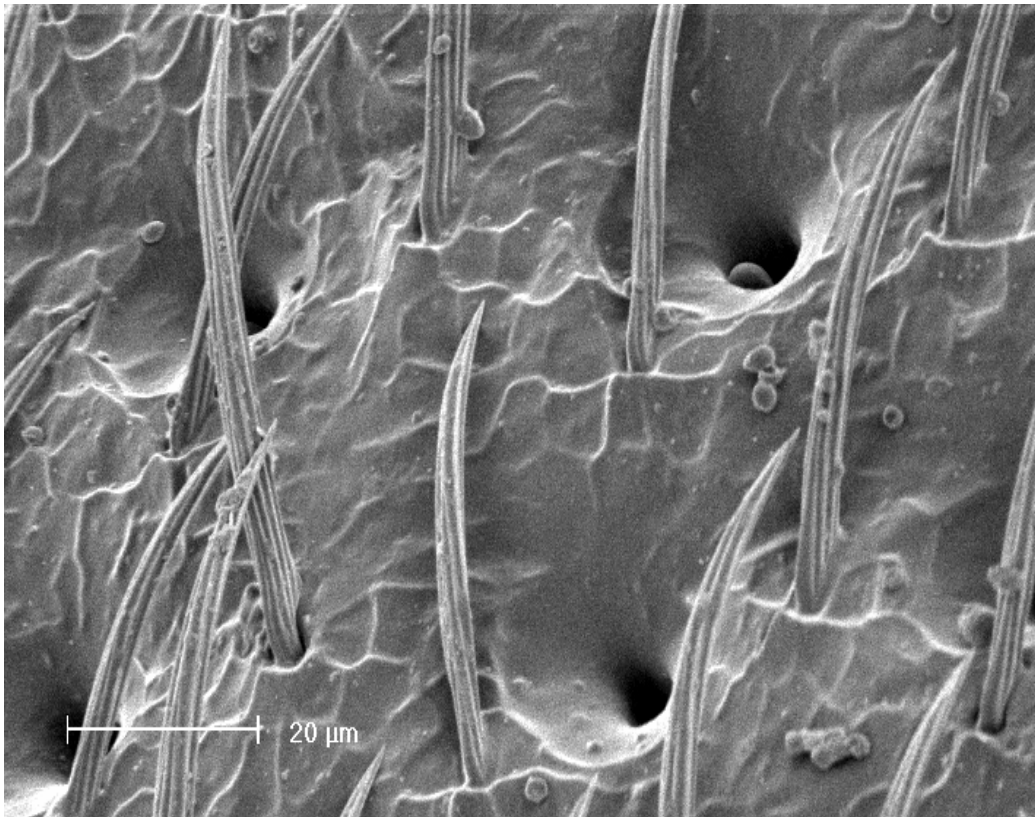
There is still some uncertainty about the precise mechanical and enzymatic steps involved in the penetration of the insect cuticle by the spore germ tube after germination of the spore but entry via the spiracles is one possible route. However, we could find no clear evidence of a difference in spore uptake between beetles kept on sprayed filter paper with and without the extra humidification. As it was not possible to draw any firm conclusions from the SEMs, other ways of tackling the problem were considered.

Figure 5. SEM of filter paper sprayed with spores.

Figure 6. SEM of a section of the leg of *O. surinamensis* showing fungal spores attached.
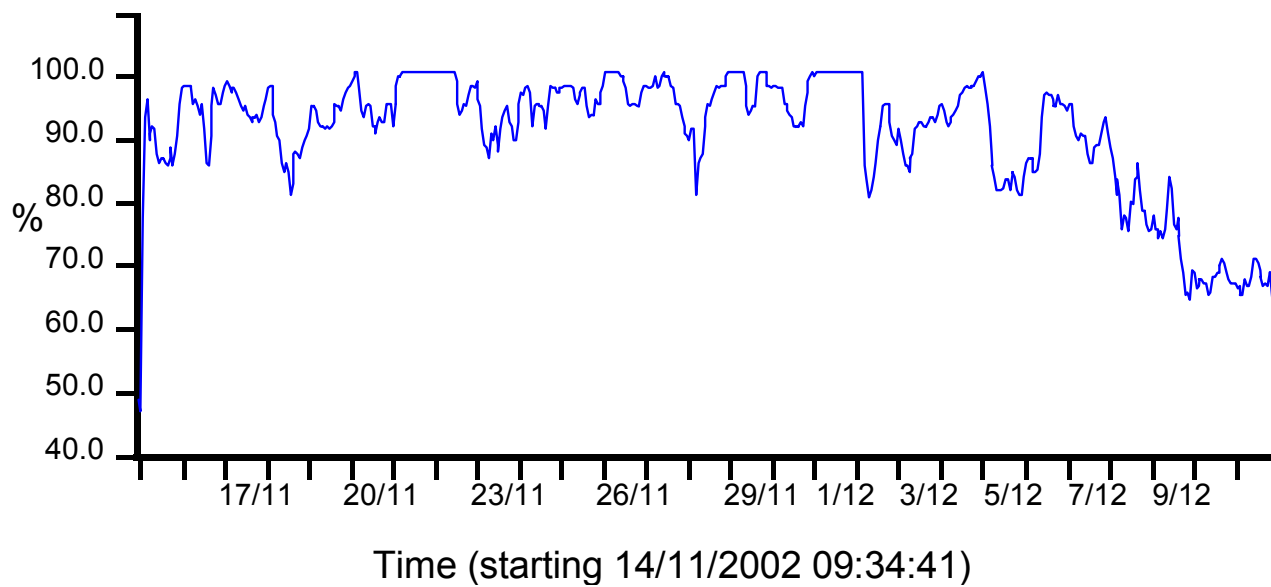
Figure 7. SEM of part of the ventral surface of the abdomen of *O. surinamensis* showing a few fungal spores in the vicinity of some spiracles.
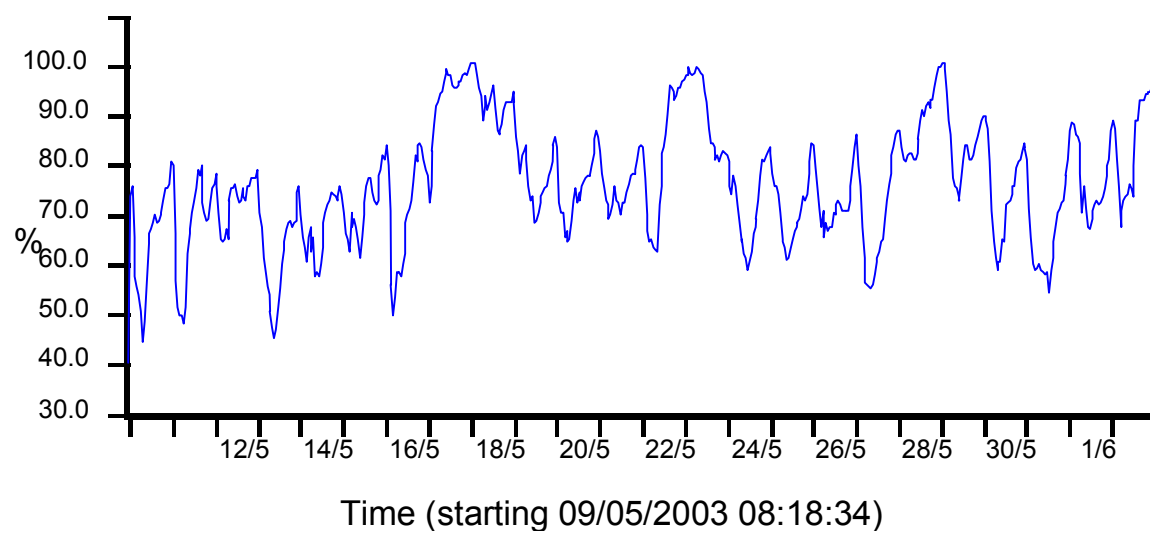
We have looked at alternative carriers to Codacide such as the industrial mineral oils, Ondina and Shellsol, as well as various mixtures of oil and water. Some oils proved to be toxic or repellent to the test insects and were discarded. So far, our original choice of a 1:4 mix of Codacide in water has proved to be the most effective, except for the problems associated with stickiness mentioned earlier.

Records in the CSL grain store using Tiny Tag data loggers were kept to provide information on the range of temperatures and RHs likely to be experienced by invertebrate pests and fungal isolates in a UK grain store over a period of 12 months. These records have shown that RHs in the range 80-100% occur frequently during the winter months, although the lower temperatures at this time of year may reduce the efficacy of fungal isolates. Figure 8 is a graph of RH during November and December 2002. Even at times of the year when we might expect to treat the structure of an empty store well before freshly harvested grain is brought in, short periods of high RH are not unusual.

Figure 9 is the graph for RH in our store during May 2003 with humidities close to 100% on several occasions. Diurnal cycles also often ensure RH is higher at night than during the day. Immediately after spraying RH will be locally higher, especially in dead spaces. However, reliance on these naturally-occurring periods of high RH may not be enough to ensure good efficacy, particularly at lower temperatures. A mycopesticide for use in grain stores would be a more attractive proposition if a fungal isolate could be selected that was more effective at lower RHs or if a formulation or application method could be found which would avoid the need for high RH in the first place.

Figure 8. Relative humidity in CSL grain store during November-December 2002.

Figure 9. Relative humidity in CSL grain store during May 2003.

MILESTONE 7. Investigate the effects of fungi added to CSL trapping devices used for invertebrate detection in storage facilities as a way of reducing pest infestation.

Laboratory studies have been conducted at CSL to determine whether healthy grain beetles can succumb to *B. bassiana* by picking up a lethal dose of spores from mycosed insects (secondary cycling). The use of PC Floor Traps as a means of disseminating spores has been assessed in laboratory arena tests.

Methods and materials

To investigate the potential for secondary cycling, 20 *O. surinamensis* Tram were placed on a filter paper in a 90 mm diameter petri dish which contained 5 dead sporulating *O. surinamensis* retained at the end of previous laboratory tests. A filter paper dampened with sterile distilled water was placed in the lid of the Petri dish for the first 24 hours after which it was removed. To provide some indication of how rapidly spores were picked up, some of the dead insects were kept in the Petri dish for the duration of the experiment while others were removed 48 hours after the experiment was set up. Mortality was assessed at 10 days. Five replicates of twenty insects were set up for each test. Tests were conducted at 20°C 70%RH.

The PC Floor Trap was used to investigate the potential of disseminating spores from trapping devices (Collins and Chambers, 2003). A 200 mm diameter aluminium ring was sealed to a 270 mm diameter filter paper using quick drying instant Polyfilla® to form an arena. A PC Floor Trap with a prototype food-volatile based multi-species lure was placed in the centre of the arena. The traps did not have the usual Fluon band and therefore insects were able to leave the trap. Traps were used either without the presence of spores or 0.025g of isolate 386243 were put into the trap. In addition, some arenas also contained a refuge consisting of a short length of aluminium tubing with mesh end caps which contained culture food. Twenty *O. surinamensis* (Tram) were released into the arena which was then covered with aluminium foil to the underside of which was adhered a 90 mm diameter filter paper moistened with sterile distilled water. After 24 hours the dampened filter paper was removed from the arena and the insects were left for an additional 24 hours. At this time the insects were removed from the arenas, noting their position (arena, trap or refuge) and transferred to 90 mm diameter Petri dishes containing a clean filter paper and a small amount of culture food. Mortality was assessed 14 days after the experiment was set up. Five replicates were used for each combination of presence or absence of fungal spores and a refuge. Tests were conducted at 20°C 70%RH.

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Results and discussion

When dead insects were removed after 48 hours, 17% of the healthy insects died within 10days. When dead insects were left in the Petri dishes for the full 10 days of the experiment, 91% of the test insects died. Secondary cycling laboratory studies at CSL have demonstrated that healthy grain beetles can succumb to *B. bassiana* by picking up a lethal dose of spores from mycosed insects. Therefore, if insects die within cracks and crevices there is the possibility that spores will be spread from sporulating to healthy insects.

The trial to examine the potential of trapping devices for spore dissemination showed that after the first 48 hours the majority of insects were in the trap, even in arenas where a refuge was present. After 14 days, there was 40% mortality of insects from arenas with traps containing spores, both with and without refuges, compared to 5% in control arenas (Table 7).

It is therefore possible for insects to pick up a lethal dose of spores from trapping devices. The potential of these insects to spread these spores to other healthy insects within the store requires further investigation.

Table 7. The potential of the PC Floor Trap for fungal spore dissemination. Mortality of *O. surinamensis* after 14 days at 20ºC 70%RH. (N=5)

Treatment	Mean % mortality ± SE
Untreated control trap	5.1 ± 3.87
Untreated control trap + refuge	3.2 ± 2.2
Trap + fungal spores	39.6 ± 4.86
Trap + fungal spores + refuge	40.0 ± 6.12

CONCLUSIONS

The work described in this project has shown that the invertebrate–specific fungus, *Beauveria bassiana*, does indeed occur naturally in UK grain storage facilities. The project has demonstrated that isolates of this fungus can give excellent control of a range of storage insect and mite pests under laboratory conditions approaching those occurring in grain stores. Secondary cycling and the use of PC Floor Traps as a means of spore dissemination have shown promise. Methodologies have been evaluated for assessing efficacy and persistence of formulated fungal isolates applied to various grain store building materials under practical UK storage conditions. Preliminary trials in the CSL grain storage facility have highlighted some

technical problems associated with the use of fungal spores in practice. To ensure good control of pests in UK grain stores, it will be essential to find ways of increasing the uptake, germination and penetration of fungal spores into the pests, for example through improved formulation and delivery systems or through the identification of isolates that are more effective at lower temperatures and relative humidities.

This project has demonstrated that fungal isolates which occur naturally in the UK have potential to control storage insects and mites. It has also shown what barriers need to be overcome for this potential to be exploited in practice. This would be of direct benefit to farmers, commercial grain store keepers and millers, and would lessen the environmental impact resulting from the use of chemical pesticides and fumigants.

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APPENDIX A

ITS sequences
Clustal W (Aiyar, 2000; Thompson *et al.,* 1994) multiple sequence alignment of approx. 600 base pair fragments for 7 *Beauveria bassiana* ITS regions, deletion mutations are indicated by dashes (-).
Forward primer region in green = ITS1F (5'-**CTTGGTCATTTAGAGGAAGTAA**-3')
Reverse primer region in red = ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

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ClustalW

    CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA Majority
    ---------+---------+---------+---------+---------+
            10        20        30        40        50
    ---------+---------+---------+---------+---------+
  1 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 51BbassianaIMI386243
  1 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 52BbassianaIMI386606
  1 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 53BbassianaIMI386367
  1 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 57BbassianaIMI387294
607 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 54BbassianaIMI386607
607 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 55BbassianaIMI386605
607 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAA 56BbassianaIMI386368


    CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA Majority
    ---------+---------+---------+---------+---------+
            60        70        80        90        100
    ---------+---------+---------+---------+---------+
 51 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA 51BbassianaIMI386243
 51 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA 52BbassianaIMI386606
 51 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA 53BbassianaIMI386367
 51 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCCAACCCTTCTGTGAA 57BbassianaIMI387294
557 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA 54BbassianaIMI386607
557 CCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAA 55BbassianaIMI386605
557 CCAGCGGAGGGATCATTACTGAGTTTTCAACTCCCAAACCCTTATGTGAA 56BbassianaIMI386368


    CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG Majority
    ---------+---------+---------+---------+---------+
            110       120       130       140       150
    ---------+---------+---------+---------+---------+
101 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 51BbassianaIMI386243
101 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 52BbassianaIMI386606
101 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 53BbassianaIMI386367
101 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 57BbassianaIMI387294
507 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 54BbassianaIMI386607
507 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGG 55BbassianaIMI386605
507 CCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCC-GGACACGGACTGG 56BbassianaIMI386368


    ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT Majority
    ---------+---------+---------+---------+---------+
            160       170       180       190       200
    ---------+---------+---------+---------+---------+
151 ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT 51BbassianaIMI386243
151 ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT 52BbassianaIMI386606
151 ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT 53BbassianaIMI386367
151 ACCAGCGGCCCGCCGGGGACC-TCAAACTCCTGTATT-CCAGCATCTTCT 57BbassianaIMI387294
457 ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT 54BbassianaIMI386607
457 ACCAGCGGCCCGCCGGGGACC-TCAAACTCTTGTATT-CCAGCATCTTCT 55BbassianaIMI386605
458 ACCAGCGGCC-GCCGGGGACCATCAAACTCTTGTATTATCAGCATCTTCT 56BbassianaIMI386368
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```
        ---------+---------+---------+---------+---------+
               210       220       230       240       250
        ---------+---------+---------+---------+---------+
199 GAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGAT 51BbassianaIMI386243
199 GAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGAT 52BbassianaIMI386606
199 GAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGAT 53BbassianaIMI386367
199 GAATACGCCGCAAGGCAAAAACAAATGAATCAAAACTTTCAACAACGGAT 57BbassianaIMI387294
409 GAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGAT 54BbassianaIMI386607
409 GAATACGCCGCAAGGCAAAA-CAAATGAATCAAAACTTTCAACAACGGAT 55BbassianaIMI386605
409 GAATACGCCGCAAGGCAAAA-CAAATAAATTAAAACTTTCAACAACGGAT 56BbassianaIMI386368

    CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATG Majority
    ---------+---------+---------+---------+---------+
               260       270       280       290       300
    ---------+---------+---------+---------+---------+
248 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG 51BbassianaIMI386243
248 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATG 52BbassianaIMI386606
248 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATG 53BbassianaIMI386367
249 CACTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAGGTAATG 57BbassianaIMI387294
360 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATG 54BbassianaIMI386607
360 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG 55BbassianaIMI386605
360 CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG 56BbassianaIMI386368

    TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC Majority
    ---------+---------+---------+---------+---------+
               310       320       330       340       350
    ---------+---------+---------+---------+---------+
298 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 51BbassianaIMI386243
298 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 52BbassianaIMI386606
298 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 53BbassianaIMI386367
299 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 57BbassianaIMI387294
310 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 54BbassianaIMI386607
310 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 55BbassianaIMI386605
310 TGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC 56BbassianaIMI386368

    CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA Majority
    ---------+---------+---------+---------+---------+
               360       370       380       390       400
    ---------+---------+---------+---------+---------+
348 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 51BbassianaIMI386243
348 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 52BbassianaIMI386606
348 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 53BbassianaIMI386367
349 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 57BbassianaIMI387294
260 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 54BbassianaIMI386607
260 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 55BbassianaIMI386605
260 CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGA 56BbassianaIMI386368

    CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC Majority
    ---------+---------+---------+---------+---------+
               410       420       430       440       450
    ---------+---------+---------+---------+---------+
398 CCTCCCCTGGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 51BbassianaIMI386243
398 CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 52BbassianaIMI386606
398 CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 53BbassianaIMI386367
399 CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 57BbassianaIMI387294
210 CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 54BbassianaIMI386607
210 CCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCC 55BbassianaIMI386605
210 CCTCCCTTTGGGGAAGTCGGCGTTGGGGACCGGCAGCACACTGCCGGCCC 56BbassianaIMI386368
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TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGC Majority
        ---------+---------+---------+---------+---------+
              460       470       480       490       500
        ---------+---------+---------+---------+---------+
    448 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGTAGTAATACAGC 51BbassianaIMI386243
    448 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGC 52BbassianaIMI386606
    448 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGC 53BbassianaIMI386367
    449 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGTAGTAATACAGC 57BbassianaIMI387294
    160 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGTAGTAATACAGC 54BbassianaIMI386607
    160 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACAGC 55BbassianaIMI386605
    160 TGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGTAGTAAACCAAC 56BbassianaIMI386368

        TCGCACCGGGACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAA Majority
        ---------+---------+---------+---------+---------+
```