PS7-26 – 6247

Factors affecting storage insect susceptibility to the entomopathogenic fungus *Beauveria bassiana*

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Abstract

There is an increasing need to examine alternatives to the use of traditional pesticides in grain storage and biological control methods have received renewed interest over the past decade. The various biological control agents that could be used include macroorganisms such as parasitoids and predators and microorganisms such as bacteria, viruses and fungi. There is increasing evidence that entomopathogenic fungi have potential for control of arthropod pest species in the storage environment. It has been demonstrated in previous studies, and further evidence obtained in this study, that storage beetle species have different susceptibilities to the entomopathogenic fungus *Beauveria bassiana*. For example, the saw-toothed grain beetle, *Oryzaephilus surinamensis* is more susceptible to *B. bassiana* than the confused flour beetle, *Tribolium confusum* at the same conidial concentration. This could be a result of a number of factors. Fungal infection of an insect occurs by a series of events. Adherence of the fungal conidia to the insect cuticle followed by germination and penetration through the cuticle are key events in the initial stages of infection. In this study *O. surinamensis* and *T. confusum* have been used as representative species to examine adherence and germination of *B. bassiana* conidia using scanning electron microscopy (SEM). It was found that there were differences between the two species in terms of both the amount of conidia found on the insect and the germination of the conidia. Fewer conidia were found on *T. confusum* compared to *O. surinamensis*. There was also little evidence of germination of the conidia on *T. confusum.* This study has provided information on the relative importance of the initial stages of infection in overall virulence and has highlighted requirements when considering formulation of conidia.

Key words: Stored product insects*; Beauveria bassiana*; Entomopathogenic fungi; Electron microscopy; Biological control.

Introduction

Cereals are an important part of both human and livestock diets. After harvesting unprocessed cereal will be stored for various lengths of time. Subsequent to the initial storage period it may also be stored at processing premises. Whilst in storage cereals are at risk to infestation by a wide range of stored product insects and mites. Traditionally, chemical pesticides have been used for the protection of stored products from attack by storage arthropods. These may be applied to the storage structure or to the commodity itself depending on the registered use. Organophosphorus (OP) pesticides have been the main group used in storage and food processing premises for prevention and control of infestations. More recently concerns have been raised with regard to these pesticides and there is an increasing

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demand to examine alternatives to their use. Biological control has received increased attention over the past few decades as an alternative to chemical treatments or as a component of integrated pest management (IPM) strategies. There have been several reviews examining the potential of biological control for protection of stored products (for example Brower et al., 1996; Cox and Wilkin, 1998; Haines, 1999; Zdárková and Fejt, 1999). Within a recently completed COST Action titled 'Biological control of pest insects and mites with special reference to Entomophthorales' (COST Action 842) Working Group IV examined "Biological control of arthropod pests in stored products" (http://cost842.csl.gov.uk/). The use of parasitoids and predators has been investigated and some of these have been sold commercially (Copping, 2004). However, widespread uptake has not occurred and this in part may be due to issues over the addition of 'contaminants' to foodstuffs and the presence of the parasitoids or predators in the end-products. Several studies have shown the potential for entomopathogens to control a range of stored product insects either alone (Searle and Doberski, 1984; Adane et al., 1996; Hidalgo et al., 1998; Moino et al., 1998; Rice and Cogburn, 1999; Meikle et al., 2001, Sheeba et al., 2001; Dal Bello et al., 2001, Cherry et al., 2005) or in combination with other control treatments such as diatomaceous earth (Lord, 2001; Akbar et al., 2004; Athanassiou, 2004; Smith et al., 2006; Vassilakos et al., 2006). Much of this work has used species and conditions more likely encountered in tropical regions. Recent research has identified the most effective naturally-occurring entomopathogenic fungi in the UK for the potential application to structures for the control of residual infestations of storage pests (Cox et al., 2004). This study also indicated that different beetle species had different susceptibilities to the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin. This work was done using a single concentration of conidia (1×10^8 conidia/ml) and it was not known whether the observed effect was due to innate differences in susceptibility between species or was an effect of the concentration used. i.e. would a higher concentration result in a high mortality for the less affected species.

Infection of an insect by entomopathogenic fungi occurs by a series of events. Some of the processes are known and understood, but there are many areas that still require clarification or investigation. The process can be divided into three parts: adhesion of the fungal spore, penetration through the cuticle and establishment within the host. Much of the work examining adherence and penetration of entomopathogenic fungal spores has been carried out using *Metarhizium anisopliae* (Metschnikoff) (see reviews of fungal pathogens by, for example St Leger, 1993; Hajek and St Leger, 1994), but some studies have also been made with *B. bassiana* (Pekrul and Grula, 1979; Neves and Alves, 2004). Attachment of several species of entomopathogenic fungi to insect cuticle has been found to be passive and non-specific (Boucias et al., 1988). It has been shown that the dry conidia of both *M. anisopliae* and *B. bassiana* are hydrophobic and suggested that hydrophobic interactions are responsible for adherence of the spore (Boucias et al, 1988; Jeffs et al., 1999). Once the conidia have adhered to the cuticle and in response to stimuli the conidia will germinate and may eventually penetrate the cuticle as a result of both mechanical force and enzymatic degradation (Charnley and St Leger, 1991). Of the various processes that may be involved in determining virulence of an isolate one of the easiest to observe is adherence and germination of the conidia on the insect cuticle. In this study we have established that there is a difference in susceptibility between species of stored product beetle and considered a possible explanation for this by examining the adherence and germination of *B. bassiana* using scanning electron microscopy.

Materials and methods

Insects

Adult insects of the following species and strains were used: *O. surinamensis* Tram, *T.*

confusum W44 and *S. granarius* Windsor. All three species were reared at 25 °C, 70 % r.h. *O. surinamensis* was cultured on a mixture of wheatfeed, rolled oats and dried de-bittered brewer's yeast mixed 5:5:1 by weight. *S. granarius* was cultured on whole wheat and *T. confusum* on a mixture of wholewheat flour and yeast mixed 20:1 by weight. Insects used were approx. zero-two weeks post adult eclosion.

Fungal isolates

The isolates of *B. bassiana* used were IMI 386243 and IMI 389521. These were isolated from insects found in UK grain stores (Cox et al, 2004). Mass production to obtain a dry spore powder of this isolate was carried out by CABI Bioscience. Production was based on a two-stage technique that included the preparation of a fungal culture in liquid medium, which was then used to inoculate the rice solid substrate (Cox et al., 2004). The dry spore powder was stored at 4-8 °C.

Treatment of insects

a) Assessment of differences in susceptibility of species

Insects were batched in groups of 20 in 7.5 x 2.5 cm glass tubes. Each batch of insects was treated individually by tipping the insects on to a glass Petri dish containing the dry conidia powder of the appropriate isolate. Individual insects were gently rolled over using fine forceps to ensure fungal conidia adhered to all parts of the insect exoskeleton. Control insects were treated in the same way but on a Petri dish without the conidia powder. The insects were then transferred to 9 cm diameter petri dishes containing a filter paper moistened with 750 µl sterile distilled water, sealed with 'Parafilm' and kept at 20 °C, 70 % rh. After 24 hours the insects were transferred to clean Petri dishes with a small amount of the appropriate foodstuff. Mortality was assessed 14 days after treatment. Dead insects were removed at the assessment period and surface sterilised by washing in 5 % sodium hypochlorite for 5 seconds followed by three rinses in sterile distilled water. The cadavers were then placed in petri dishes on filter papers moistened with sterile distilled water; after 5 days at 20 °C, 70 % rh they were examined for external sporulation of fungus to confirm that death was due to mycosis.

b) Assessment of conidial attachment and germination

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

SEM examination

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

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

Results

The pathogenicity of two different isolates of *B. bassiana*, IMI 386243 and IMI 389521 was examined with three different insect species using a high challenge test in which a large amount of conidia was in contact with the surface of the insect. High levels of mortality were recorded for *O. surinamensis*, but much lower mortality levels were found for *S. granarius* and these were lower again *for T. confusum* (Figure 1). Control mortality was less than 5 % for all three species. For each individual species there was little difference between the efficacy of the two different *B. bassiana* isolates.

One possible cause for the differences observed could be related to the adherence and germination of the conidia on the insect cuticle. This was examined using scanning electron microscopy (SEM). Three different body areas were examined after treatment with a conidial suspension for *O. surinamensis* and *T. confusum*. It was found that there were differences between the two species in terms of both the number of conidia found and the number of germinating conidia seen (Figure 2 and Figure 3 a-d). Examination at all three time periods post-treatment showed that conidia were present in greater numbers on the cuticle of *O. surinamensis* in all three body regions examined compared to the same regions for *T. confusum*. Observations at 24, 48 and 72 h post-treatment showed that some conidia on the cuticle of *O. surinamensis* had germinated, but this was observed for less than 5% of the conidia present. Germinating conidia were not observed on the cuticle of *T. confusum* until 48 h post-treatment. The germ tubes seen were much shorter than those observed on *O. surinamensis* and fewer conidia were observed to show any sign of germination. It was

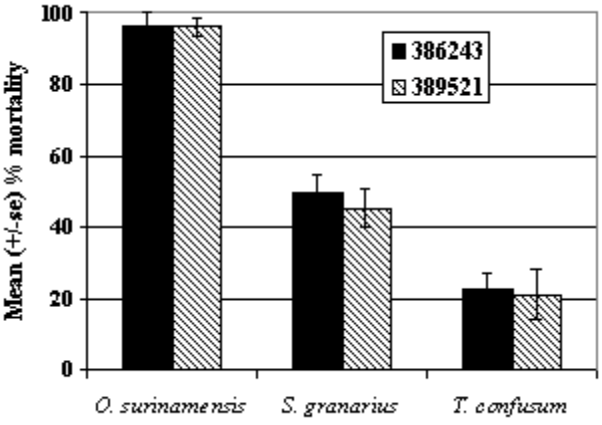


Figure 1. Mean % mortality (± SE) of three species of storage beetle 14 days after treatment with two isolates of *B. bassiana* dry spore powder.

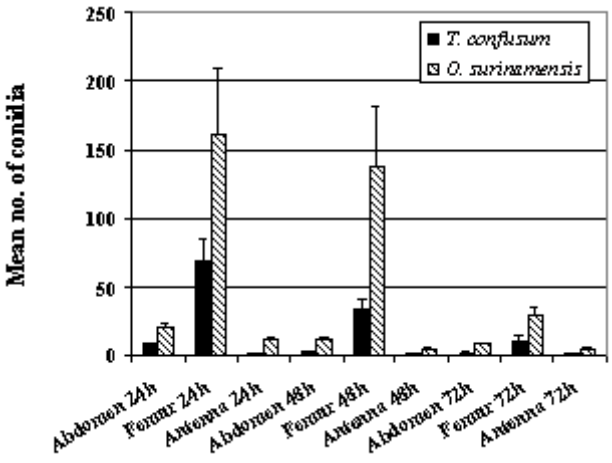


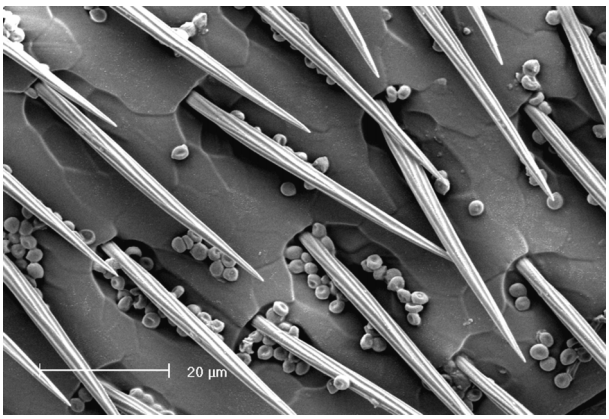
Figure 2. Examination of conidial attachment at different time periods post-treatment.

found that for both species the number of conidia decreased with increasing time post-treatment.

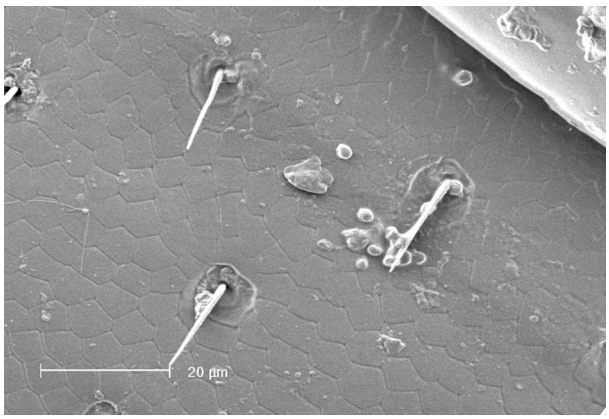
Discussion

The 'high challenge' test showed that the differences in storage beetle susceptibility to *B. bassiana* could not be explained as a function of the concentration of conidia used. The results confirmed those from a previous study (Cox et al., 2004) that had used a concentration of 1 x 10^8 conidia/ml. An electron microscope study was undertaken to determine whether differences in adherence and germination of the conidia on the cuticle of *O. surinamensis* and *T. confusum* could explain the apparent differences. This study showed that quantitative and qualitative differences could be observed between the two species. *O. surinamensis* had a greater number of conidia adhering to the cuticle at each of the post-treatment periods. This species had a much greater number of setae, particularly on the ventral abdomen, and the presence of these may have assisted with adherence of the conidia. Germinating conidia were observed more frequently on the cuticle of *O. surinamensis* than for *T. confusum*. There are several possible reasons for this 1) the setae of *O. surinamensis* may trap air near the surface of the cuticle which, as a result of cuticular and respiratory transpiration processes, may contain higher levels

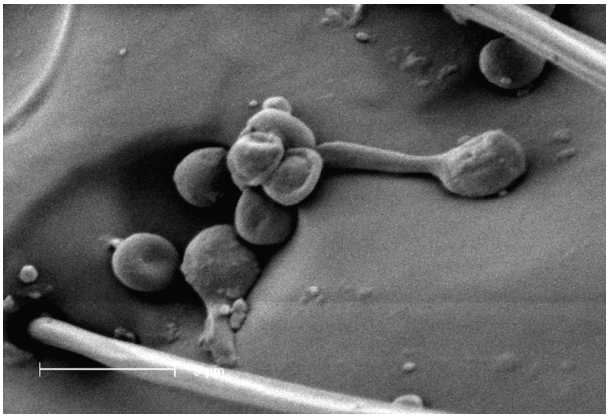
a) *O. surinamensis* 24 hr



b) *T. confusum* 24 hr



c) *O. surinamensis* 72 hr



d) *T. confusum* 72 hr

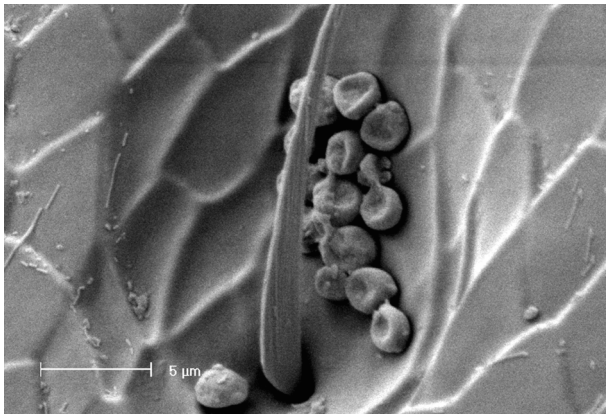


Figure 3. Attachment and germination of conidia on the ventral abdomen of *O. surinamensis* and *T. confusum.*

of moisture. This would provide more favourable conditions for germination of the conidia. 2) The cuticular lipid profiles will differ between the two species. It is possible that the cuticle of *T. confusum* contains a substance that is inhibitory for conidia germination. It has been shown that cuticular hydrocarbons can either promote or inhibit conidial germination (St Leger, 1991 and references therein). *Tribolium* species are also known to produce defensive quinones and it is possible that these chemicals may also inhibit germination. Inhibition of yeast and bacterial growth by the defensive secretions from *Tribolium* spp. has been shown (Prendeville and Stevens, 2002). Current studies are investigating these possibilities.

The number of conidia found on both species decreased over time. This could be as a result of grooming activities. From this it would appear that conidia that have not germinated and penetrated the cuticle within the first 24-48 hours are unlikely to remain on the cuticle and play a role in the infection process.

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

Acknowledgements

This project is sponsored by Defra through the Sustainable Arable LINK Programme with support from the UK Home Grown Cereals Authority and nine other industrial partners. Research partners are the Central Science Laboratory and CABI Bioscience. Provision of the conidia by CABI Bioscience, UK is gratefully acknowledged.

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