White rice and pearled barley mass cultured *Beauveria bassiana* and its pathogenicity toward *Choristoneura fumiferana*

by

Sheila White

A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia,
in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Applied Science

June 21, 2011, Halifax, Nova Scotia

Approved:	Dr. Doug Strongman Co-Supervisor Department of Biology
Approved:	Dr. Barry Hicks Co-Supervisor College of the North Atlantic
Approved:	Dr. Peggy Dixon External Examiner Atlantic Cool Climate Crop Research Centre
Approved:	Dr. Madine Vanderplaat Supervisory Committee Department of Sociology
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Approved:	Dr. Jeremy Lundholm Graduate Studies Representative
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To my Grandparents Terrence and Florence White, and Arnold and Mary Pike

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Abstract

An assay screening five isolates of the entomopathogenic fungus *Beauveria bassiana* from various geographic and host origins, found the following strains most pathogenic toward 4th, 5th and 6th instars of Eastern Spruce Budworm (ESB): 1756 (sawfly larva, Nova Scotia); ARSEF1850 (Western Spruce Budworm larva, British Columbia); and DAOM196480 (ESB larva, N.B.). The ARSEF and DAOM strains were mass cultured on long grain white rice and pearled barley, and the pathogenicity of the resultant product towards 6th instars of ESB larvae was assessed in a bioassay. ARSEF out-performed DAOM in the mass culture trials, and white rice and barley were suitable production substrates. With the exception of one inoculum that consistently caused lower mortality, efficacy of the commercial product BotaniGard (GHA strain)-a positive control in the bioassay, and the barley, rice, and agar grown isolates toward ESB, was similar. The GHA strain and agar grown isolates demonstrated slightly higher virulence however.

Introduction

Developing a protocol for mass culturing pathogenic *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) conidia was the principal aim of this project. The larger scope, within which this objective was sought, is to eventually develop this fungus into a mycoinsecticide targeted toward north-temperate forest Lepidoptera, in particular Eastern Spruce Budworm (ESB) *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). This study represents the earliest of the enormous research needed in pursuit of such a goal. Corresponding with Integrated Pest Management (IPM) ideology, the developed mycoinsecticide would be an environmentally safe, effective, biological control that could be applied with, or as an alternative, to controls currently available. A naturally occurring, primarily soil-dwelling entomopathogenic fungus, *B. bassiana* has been widely studied, in particular its biology, hosts, impacts on non-target organisms and safety towards vertebrates (De Hoog, 1972; MacLeod, 1954; Steinhaus, 1949; Zimmerman, 2007). Exhibiting a percutaneous infection pathway, deemed safe for vertebrates, possibly host specific, and synergistic when applied with some other entomopathogenic organisms and chemical controls (Feng et al., 2004; Ma et al., 2008; Shi and Feng, 2006), *B. bassiana* would make an exceptional mycoinsecticide if an efficacious mass production system and suitable formulation could be established for it in North America.

Eastern Spruce Budworm is a pest principally of balsam fir *Abies balsamea* (L.) Mill (Pinaceae), white spruce *Picea glauca* (Moench) Voss. (Pinaceae), red spruce *Picea rubens* Sarg. (Pinaceae), and black spruce *Picea mariana* (Mill.), B.S.P (Pinaceae)

(Hudak, 1981) but will also attack Eastern larch *Larix laricina* (Du Roi) K. Koch (Pinaceae) and Eastern hemlock *Tsuga canadensis* (L.) Carr. (Pinaceae). It is considered one of the most destructive insects of North America, to which it is native (Hudak, 1981). Outbreaks of this pest, considered by Blais et al. (1981) and others (Blais, 1984; Baskerville, 1975; Crawford and Jennings, 1989) as a natural component of fir-spruce stand dynamics and elemental to the evolution of North American forests, have been recorded as early as the 1700s (Blais, 1965; Boulanger and Arseneault, 2004; Krause, 1997). Large-scale outbreaks can destroy greater than 50% of infested fir-spruce stands, and forest hectares in the tens of millions were lost in the 1970s and 1980s in Eastern Canada during the last outbreak (Health Canada, 2005; MacLean, 1984). Outbreaks of ESB recur every 30-40 years (Boulanger and Arseneault, 2004; Royama, 1984).

Control of ESB from the 1950s to the mid 1960s was mainly by the wide-spectrum organochloride dichlorodiphenyltrichloroethane (DDT) that was banned in the U.S in 1972 (U.S. Environmental Protection Agency, 2011), and in Canada in 1985 (Environment Canada, 2010). The organophosphate fenitrothion was used as well (Department of Natural Resources (D.N.R), 1976) but large-scale applications were terminated in 1998 (Health Canada, 2005). The carbamate aminocarb was used but was withdrawn from the market in 1987 (Health Canada, 2005). The insecticides acephate, carbaryl, phosphamidon and trichlorfon among others have also been applied to varying extents (Armstrong, 1984). The use of chemicals for insect pest control has given rise to insecticide resistance, outbreaks of secondary pests usually held in check by natural predators, safety hazards for humans and domestic animals, contamination of ground

water, and decreased biodiversity (Lacey et al., 2001). For these reasons, and because of smaller outdated toxicological databases (Health Canada, 2005), low efficacy, and high cost, these chemical products are becoming unacceptable for large-scale use, and increasingly there is a focus on finding alternatives for forest pest control (Bravo and Soberón, 2008; Health Canada, 2005; Jackson, 1997).

As Lacey et al. (2001) point out, there is potential for microbial agents to fill the void left by these phased out chemicals. This was first realized with the development of the natural soil bacterium, *Bacillus thuringiensis* (Bacillales: Bacillaceae) (*Bt*). Introduced to forestry in the 1980s, today it is the foremost agent used in spruce budworm management. Because it is less persistent, has no contact toxicity and is sunlight degradable, *Bt* has a reduced environmental impact compared to chemical controls (Blais, 1976; Health Canada, 2005). Tebufenozide (Mimic®), a synthetic insect growth regulator, sometimes used instead of *Bt*, offers similar environmental protection. While these are promising, neither is ideal since they both rely exclusively on host feeding behavior (Schoenmaker et al., 2001). If a sub-lethal dose is ingested, larvae can recover and still cause defoliation. Another concern is that most trials on *Bt* for budworm control took place during the mid-1980s and 1990s, when budworm populations were declining, and it produced lower larval mortalities than chemical insecticides (Health Canada, 2005). It is not known whether *Bt* will be sufficiently efficacious in future outbreaks when epidemic populations are reached (Health Canada, 2005). There is also a real possibility that *Bt* and other pest controls will fail us in the future because of resistance development (Gahan et al., 2001; Heckel et al., 2007).

Great effort has been devoted to the possibility of controlling pests biologically, and

an enormous spectrum of information related to these efforts exists. Documentation with respect to where, when, by whom and the frequency of use of biological control worldwide is fragmented (Gelernter, 2007). It is clear that despite the quest for alternative controls, over the last three decades no novel insecticides (beyond *Bt*) have been developed for spruce budworm and other forest lepidopteran pests. The effectiveness of pheromones for spruce budworm monitoring and management has been studied but conflicting results from trials in the 1970s, 80s and into the 1990s means that their potential is not known (Health Canada, 2005). Research on *Trichogramma minutum* Riley (Hymenoptera: Trichogrammatidae) a parasitoid that kills spruce budworm eggs has achieved promising results, while trials on other microbes: fungi, bacteria, microsporidan protozoa and viruses, were indeterminate (D.N.R, 1976; Health Canada, 2005). Pathogenic protozoa, bacteria and viruses typically enter the host through openings such as spiracles or wounds and by ingestion; entomopathogenic fungi can invade the host in these ways, but ultimately reach the haemocoel through the cuticle (Ferron, 1981; Furguson, 1992; Jackson, 1997). Attacking the host integument, *B. bassiana* conidia attach to, germinate on and penetrate the cuticle with a germ tube. Entering the haemolymph, hypal growth continues eventually defeating host responses and immune defense reactions, and death results from organ destruction and/or toxin production (Furguson, 1992). The pathogen proliferates within the body forming yeast-like vegetative blastospores, and after host death, hyphae emerge from the cadaver to produce conidia for further disease spread (Ferguson, 1992; Ferron, 1981; Zimmerman, 2007).

As well as the infection mode of *B. bassiana* and contributing further to its promise as an active mycoinsecticide agent, is its broad host range when used alone and

in combination. Feng et al. (2004) found it useful to control the false-eye leafhopper *Empoasca vitis* (Göthe) (Homoptera: Cicadellidae) in China, and emphasized a synergistic effect when used with Imidacloprid, a chemical insecticide. A study done by Irigaray et al. (2003) on Naturalis-L®, a conidial formulation of *B. bassiana,* found it efficacious for controlling the twospotted spider mite *Tetranchus urticae* Koch (Acari: Tetranychidae). Ma et al. (2008) reported *B. bassiana* effective to control the Asiatic corn borer *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae), and additive effects when applied with *Bt*. Shi and Feng (2006) reported its efficacy for controlling the citrus red mite *Panonychus citri* (McGregor) (Acari: Tetranychidae), when applied alone and with pyridaben, a pyridazinone-derived acaricide. *B. bassiana* is reportedly effective against the diamond back moth (DBM) *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Godonou et al., 2009; Shelton et al., 1998). In a comparative study on Mycotrol® and Bioblast®, commercial formulations of *B. bassiana* and *Metarhizium anisopliae* (Metsch) Sorokin (Hypocreales: Clavicipitaceae) respectively, and unformulated blastospores of *Paecilomyces fumosoroseus* (Wize) (Eurotiales: Trichocomaceae), Mycotrol® most effectively controlled the DBM population proving as useful as *Bt* (Shelton et al., 1998). Tefera and Pringle (2003) found *B. bassiana* effective against the stem borer *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) when larvae were exposed to an infective spray, the dip method and conidia treated leaves. Efficacy of *B. bassiana* against the satin moth *Leucoma salicis* (L.) (Lepidoptera: Lymantriidae) has also been verified by Zurek and Keddie (2000).

Bioassays, the tools for assessing and comparing virulence among isolates of entomopathogenic fungi toward various hosts, are also used to determine host range,

epizootic potential and performance under field conditions (Butt and Goettel, 2000). The vast range of these pathogens, and of the hosts toward which they are effective and that vary in their environmental needs, means that no standardized bioassay systems exist (Goettel and Inglis, 1997). So they must be designed based on host-pathogen combinations and the objectives of each study. The salient components of bioassays are the presentation of a precise dose to the host, the incubation of infected insects, and mortality assessments on a daily basis (Butt and Goettel, 2000; Goettel and Inglis, 1997).

Enumeration of inocula propagules is imperative, and is typically accomplished with direct counts using a haemocytometer, as described by Goettel and Inglis (1997). In this method an estimation of propagules per ml is obtained, and the original suspension is adjusted to the target concentration. Inoculation of entomopathogenic fungi is typically by topical application to the integument, while injection or oral administration can also be used in studies where the cuticular barrier is not of particular importance (Butt and Goettel, 2000). As described by Goettel and Inglis (1997) and Butt and Goettel (2000), direct methods such as dusting, dipping or spraying can be used for topical application, or indirect methods, such as the use of baits. These methods have advantages and disadvantages, and have been used with varying degrees of success. Deposition of inoculum to the host directly with a droplet was the method selected by Holder et al. (2007) as well. Cottrell and Shapiro-Ilan (2003), and James and Lighthart (1994) on the other hand, have capitalized on the dip method. James et al. (1998), and Tefera and Pringle (2003), have used indirect methods of inoculation.

After being inoculated, insects are incubated in controlled environmental conditions maintained with suitable chambers or incubators (Goettel and Inglis, 1997).

The feeding regime and incubation method are tailored to the host insect needs and bioassay objectives, but should favor survival of non-inoculated control insects (Butt and Goettel, 2000). Mycosis assessments of the incubating insects are typically made daily for 1-2 weeks, as determined by the rapidity with which the pathogen acts (Butt and Goettel, 2000). Mycosis verification is determined by incubating each cadaver in a high moisture environment (a Petri dish containing moistened filter paper), which encourages fungal colonization and sporulation on the cuticle (Goettel and Inglis, 1997). As Butt and Goettel (2000) point out, control insect cadavers should also be incubated in moisture chambers, to determine residual infection levels or if accidental contamination occurred.

The infection mode of entomopathogenic fungi as exemplified by *B. bassiana* is highly exploitable for insect pest control. With their vast host ranges and the environmental protection they offer, it is no surprise that the role of mycoinsecticides in IMP is increasing (Li et al., 2010). As Faria and Wraight (2007) point out, over 170 mycoinsecticides and mycoacaricides have been developed in more than 80 countries worldwide since the 1960s. Of these products, 75% are registered, undergoing registration or available commercially (sometimes without registration), 15% are discontinued and the status of the final 10% is unknown. *Beauveria bassiana* described by Safavi et al. (2007) as "well-known and cosmopolitan" is among the most intensively investigated of the entompathogenic fungi as a promising biocontrol agent (Butt et al., 2001; Boucias and Pendland, 1998; Feng et al., 1994). It forms the base of over a third of the mycoinsecticides listed by Faria and Wraight (2007), a total of 58 products. Of these, 45 are registered, undergoing registration or available, 9 are no longer available or were never submitted for registration, and the status of the remaining 4 products is not known.

Other important species are *M. anisopliae*, *Isaria fumosorosea* (Wize) and *Beauveria brongniartii* (Sacc.) (Hypocreales: Cordycipitaceae) (Faria and Wraight, 2007).

The earliest record of *B. bassiana* dates back to *Bb* infected silkworms that were described by an ancient pharmacologist in a famous book (Bencao Gangmu-Book of Medicinal Herbs) initially published in 1596 and reedited in 2006 (Li et al., 2010). The early history of this pathogen however began in 1835 with Agistno Bassi making the same observation, and conducting the first infection experiments (Zimmerman, 2007). The marked increase in research and development efforts into this pathogen in the many years following resulted in the first successful *B. bassiana* mycoinsecticide (Boverin) in 1965 in the former U.S.S.R (Feng et al., 1994). Although incomplete, inconsistent and sometimes conflicting literature is an impediment to determining the status of microbial control, it is clear that countries in Asia, Latin America and Eastern Europe have accounted for the greatest use of fungal pathogens (Faria and Wraight, 2007). China has been particularly important to the development and use of *B. bassiana,* successfully culturing it in metric tons to apply to some 0.8-1.3 million ha in the 1980s, for protection against forest and farm insect pests (mainly pine caterpillars and the Asian corn borer) (Feng et al., 1994; Li, 2007; Li et al., 2010). Spray applications were of pure conidia, conidia dispersed in emulsifiable oils, unformulated culture powders containing conidia and substrates, and conidial suspensions made with milled and non-milled culture powders. Other novel application techniques such as conidial fireworks, use of explosives and the release of fungus-inoculated caterpillars were also developed around this time (Li, 2007; Li et al., 2010). While China has had one of the largest, most successful microbial control programs worldwide (Li, 2007), government funding which fueled these

operations has declined in recent years (Feng, 2003).

Beginning with selecting a fungal isolate with rapid growth, abundant sporulation, and sufficiently high pathogenicity to the target pest, a feasible production system must be low-cost and provide high yields of viable, virulent, and persistent propagules (Feng et al., 1994; Jackson, 1997; Jenkins et al., 1998). Nearly 200 strains of *B. bassiana* from various geographic regions for example, were screened prior to the recommendation of the optimal strains (113N-278 and 124-R) for incorporation into Boverin (Feng et al., 1994). The majority of mycoinsecticides depend on the production of aerial conidia, since infections of insects are initiated by conidial contact with host insect cuticles (Faria and Wraight, 2007; Wu et al., 2008). As well as aerial conidia however, *B. bassiana* also produce mono-nucleated in vitro blastospores and submerged conidia (Holder et al., 2007) and mass production of all these cell forms has been studied (Feng et al, 1994; Li, 2007 and Li et al., 2010).

Diphasic (also 'biphasic'), submerged (also called 'liquid culture') fermentation, and solid substrate production techniques, as reviewed by Feng et al. (1994) are commonly researched in an effort to produce infective *B. bassiana* propagules and several media sources have been tested in conjunction with these methods. Diphasic fermentation capitalizes on both solid and liquid media for the production of aerial conidia. Vegetative mycelium is produced in liquid culture, and subsequently conidiates on an inert or nutritive substrate (Feng et al., 1994). With submerged fermentation, blastospores, or a mixture of blastospores and conidia, form in yeast-like fashion in liquid culture as in the insect haemocoel; these are considered functionally identical to short hyphal cells. The production of conidia in submerged culture occurs with this same basic method. Solid

substrate methods typically involve inoculating an inert or nutritive substrate with a spore suspension for the subsequent multiplication of the fungus there (Feng et al., 1994; Vega et al., 2003; Wu et al., 2008).

Feng et al. (1994) stated in a review paper that blastospores can be highly infective when applied to insect hosts, but are impractical for large-scale use because of loss of viability during harvest, formulation, and storage, even when conditions are controlled. More recently, Vega et al. (2003) confirmed that blastospores are generally not amenable to simple drying techniques, and tend to die more rapidly during storage than aerial conidia. Feng et al. (1994) also point to the high cost of submerged fermentation, which is a drawback for large-scale operations. This method has been discontinued in China for these reasons (Li et al., 2010); and producing conidia in submerged culture was also considered impractical because of low yields (Feng et al., 1994). Goettel (1984) also pointed out that submerged conidia are usually less virulent and have shorter half-lives than surface produced conidia.

Small-scale success has been achieved with the production of *B. bassiana* conidia on solid substrates in the absence of a liquid fermentation step. Goettel (1984) described a simple procedure for this with wheat bran, and many researchers have investigated adapted forms of these methods. Li et al. (2010) describes a three-stage solid substrate production system developed in China in the 1970s that had some success but ultimately failed due to inconsistent yields. And Mycotech Corporation (U.S) developed a solid culture process for *B. bassiana* production as well (Feng et al., 1994). The production of powdery *B. bassiana* on rice using similar methods to Goettel (1984) was reported by Feng et al. (1994); and a host of other substrates have been investigated. Ibrahim and Low

(1993) experimented with grated coconut flesh, mature papaya fruit, tapioca root, sweet potato, tubers and rice grains. Mazumder et al. (1995) investigated press mud, bagacillo (sugar mill waste), riverbed sand, sawdust, and rice husk. In each of these studies the density of *B. bassiana* conidia produced was variable.

Automated diphasic technology developed in China in the 1980s was thought to be the most promising for practical production of *B. bassiana* (Feng et al., 1994), and variations of these methods persist in large-scale operations today. Using machinery and capitalizing on cheap, plentiful materials for growth substrates, production of high-quality conidial powder is simple, cheap and safe using this technology. Improvements to this basic system to increase conidial yield, reduce contamination and improve mechanization are continually being sought (Li et al., 2010). A modern variation of this system (utilizing large liquid and solid fermenters) allowed privately owned companies in China to produce and register six *B. bassiana* based products in 2008 (Li et al., 2010). Increasingly the term 'solid substrate fermentation' is used as a reflection of new technologies (Ye et al., 2006). Jackson (1997) maintains that Mycotrol, a *B. bassiana* product of Mycotech Corporation (U.S) is produced using solid substrate fermentation. This is likely the method which most manufacturers listed by Faria and Wraight (2007) that rely on aerial conidia, use. Diphasic principles for producing *B. bassiana* are constantly being researched on a smaller scale as well in search for novel media and protocols to promote mass sporulation of various isolates (Nelson et al., 1996; Karanja et al., 2011).

As mentioned previously successful mass production of an entomopathogenic fungus is preceded by the selection of an isolate with, among other qualities, adequate sporulation. Found in (minimally) 707 host insect species worldwide, (521 genera; 149

families, and 15 orders), and isolated from a number of other sources (Ormond et al., 2010; Zimmerman, 2007), *B. bassiana* is a species complex, within which considerable natural genetic variability and heterogenicity is found (Devi et al., 2006; Maurer et al., 1997; Padmavathi et al., 2003). While tremendous effort toward elucidating the environmental factors that affect conidiation has been invested, knowledge is still lacking, and gene regulation of this process is not understood. For instance, it has been shown that isolates have either suppressed vegetative growth and abundant sporulation, or the reverse (Feng et al., 1994), but the process regulating the switch between vegetative growth and sporulation is not known (Zhang et al., 2009). It is well documented that humidity, cultural media, temperature and light period affect conidiation of entomopathogenic fungi (Arthurs and Thomas, 2001; Onofre et al., 2001; Rosa et al., 2004), but exact mechanisms as to how they respond to environmental stimuli are yet to be determined (Zhang et al., 2009). Wu et al. (2008) point out that conidiation of *B. bassiana* reflects a complex and little understood process involving the stage- and cell-type-specific expression of hundreds of genes. Considering all of this together, mass culturing protocols for optimum sporulation of *B. bassiana* are certain to vary among isolate genotypes, and in accordance with how a specific genotype regulates an isolate's response to environmental stimuli. Due to the genetic diversity among *B. bassiana* isolates, coupled with a lack of understanding of the genetic mechanism that drives conidiation, such optimization can only be established through experimentation. As Nelson et al., (1996) point out, for each new strain (of an entomopathogenic fungus), optimal conditions required for large-scale production differ and need to be tested.

Nutritional conditions to promote sporulation of entomopathogenic fungi have been

described to varying extents. Jackson (1997) emphasizes the importance of significant biomass accumulation for optimal spore yield, and indicates this yield to be a function of the endogenous nutrients accumulated by the fungus during vegetative growth. He suggests that a production medium can be formulated based on the nutritional framework of a first developed and optimized defined medium. The nutritional components of the defined medium are replaced with low-cost, complex substrates in the production medium.

In studies similar to this one, to establish working/starting cultures of entomopathogenic fungi, researchers usually inoculate them on Sabouraud's Dextrose or potato dextrose agar, sometimes fortified with yeast extract (Bateman et al, 2004a; Damir, 2006; Rangel et al., 2005; Safavi et al., 2007). Bateman et al., (2004a) indicate that carbohydrate and nitrogen supplies are imperative for the broth element when diphasic methods are used. Carbohydrates provide energy; and nitrogen, supplied in protein, or by the inclusion of amino acids or inorganic nitrogen from which protein can be synthesized, is crucial for growth. Several sugars are maintained to satisfy the carbon requirement: sucrose, glucose, D-glucose or dextrose, fructose or maltose; and yeast, peptones, meat digests, or inorganic nitrogen suffice for the nitrogen source. Inorganic nitrogen tends however to be less easily utilized by fungi and can result in limited growth. As for sugars, glucose is the most utilizable carbon source for fungi, while others are less preferred (Braga et al., 1999; Hallsworth and Morgan, 1994; Rangel et al., 2006; Shah et al., 2005). The solid substrate in diphasic methods functions principally as a physical support for the fungus to produce aerial conidia; and its structure is thought to be as, or more important, than its nutritional content (Bateman et al., 2004a). Ideal substrates have a high surface

area to volume ratio with the ability to keep individual particles separated. This facilitates air exchange and supports conidial formation (Bateman et al., 2004a). With these basic nutritional requirements in mind, alterations in spore morphological and physiological characteristics which can lead to improved mycoinsecticides can be achieved by manipulating carbon and nitrogen sources, trace metals, vitamins, carbon loading and C:N ratios (Jackson, 1997; Ibrahim et al., 2002). In the search for optimized defined and production media, many researchers have studied such alterations and have assessed their impact on spore yield and fitness.

The main objective of my study was to produce large quantities of *B. bassiana* conidia from isolates of Eastern and Western Spruce Budworm on long grain white rice and pearled barley, that were virulent towards Eastern Spruce Budworm. Diphasic mass production techniques adapted mainly from the work of Bateman et al. (2004a), but in consultation with many sources, were used. Direct manipulations of nutrients as described above were not done in this work, as the main goal was to obtain information about the potential of pearled barley and long grain white rice as growth substrates for *B bassiana*.

Preface

Methods used in the main analyses were chosen based on preparatory experiments at Saint Mary's University, Halifax, Nova Scotia (Winter 2010), and the College of the North Atlantic in Carbonear, Newfoundland (Spring, 2010). Associated and continuous with the main studies, these initial experiments denoted '***Preparatory***' investigations, are described and the findings given, at the outset of the Materials and Methods, and Results sections. The main studies indicated as '***Principal***' investigations described subsequently, were completed at the College in Carbonear.

Materials and Methods

Preparatory Investigation

Beauveria bassiana isolate sporulation and pathogenicity toward 4th, 5th and 6th instars of *Choristoneura fumiferana* larvae:

Rationale

In addition to *B. bassiana* isolate growth experiments, preliminary bioassays were undertaken to select the most pathogenic isolates (among those available) toward ESB for subsequent mass culture trials, and to determine if susceptibility to this pathogen differed among the instars (4^{th}, 5^{th}, 6^{th}) investigated. The experiments were preparatory in nature, designed to develop standard procedures for subsequent bioassays that would investigate *B. bassiana* cultured on grains. Methodology irregularities, for example variable inocula concentrations and preparation methods, are an aspect of this rationale, while consistency was affected for incidental reasons as well. Establishing a stable sample size for example was problematic due to rearing logistics. This is because it was necessary to stagger larval rearing and isolate cultures, to ensure viable and sufficient numbers of $4^{th}/5^{th}$, and 6^{th} instars for each isolate simultaneously, and coinciding with the established incubation period for the fungal isolates (18 days). Rearing three instar groups (4^{th}, 5^{th} and 6^{th}) was impractical due to the short duration of the 4^{th} and 5^{th} instar in rearing, so the 4^{th} and 5^{th} instars were combined in a single group. A minimum of 20 larvae per treatment was attempted but samples sizes varied between the instar groups; and some treatments were excluded when rearing numbers were exceptionally low. Contributing to this variation was the ability of larvae (primarily the 4^{th} and 5^{th} instars due to their small size) to escape the diet cups through any gaps about the lid. This was a problem throughout the

experiments, and aside from monitoring the situation and replacing lids as necessary, the only solution was to reserve as many replacement larvae as possible for each instar group when the treatments were set up. However, an escaped, inoculated larva was considered displaced and contributed to a lower sample size.

Saint Mary's University (SMU), Halifax, Nova Scotia (Spring, 2010)

In a qualitative analysis investigating sporulation and conducted in two replicates, daily macroscopic and microscopic examinations of *B. bassiana* cultures were made. The cultures were established from five available isolates (Table 1) by streaking sub-cultured conidia on Sabouraud's Dextrose Agar (SDA) (Appendix A1) (Difco, VWR International, Mississauga, ON), in 9 cm Petri dishes (Table 1). The commercial *B. bassiana* strain in BotaniGard (GHA) (Table 1) was not assessed in these experiments. Because it is a wettable powder and contains ingredients additional to *B. bassiana* spores, it is not conducive to being cultured on agar. Aseptic technique (Bateman et al., 2004c) was used when fungal cultures were established, whether on agar, or as in subsequent experiments, in broth or on grain. These conditions were also maintained for all inocula preparation procedures, and for contamination checks performed throughout all experiments. The cultures were kept in an incubator maintained at 25°C (Goettel and Inglis, 1997; Jenkins et al., 1998). Mean recorded conditions were 24.4 ± 0.0°C and 17.2 ± 3.8% RH (experiment 1); 24.3 ± 0.1°C and 14.6 ± 4.6% RH (experiment 2). Following this investigation the isolates were sub-cultured in 2 ml cryogenic vials containing 25 inoculating beads each (Inter Medico, Markham, ON), and were stored in a -70°C freezer (Humber, 1997). Fresh cultures were later established from these cryo-preservations on

SDA, and using true replication, the isolates were assayed against 4th, 5th and 6th instars of *C. fumiferana.*

Table 1. *Beauveria bassiana* isolates used in mass production and pathogenicity trials on Eastern Spruce Budworm (*Choristoneura fumiferana*).

***B. bassiana* isolate accesssion #**	**Host**	**Geographic origin and date isolated**
ARSEF1850	*Choristoneura occidentalis* (Lepidoptera: Tortricidae)	Terrace, B.C (06/83)
DAOM196480	*Choristoneura fumiferana* (Lepidoptera: Tortricidae)	Fredericton, N.B (07/86)
B911	*Croesia curvalana* (Lepidoptera: Tortricidae)	Ontario (06/92)
2082	Coleoptera: Chrysomelidae	Micklefield, N.S. (07/26/91)
1756	*Neopareophora sp. (*Hymenoptera: Symphyta)	Parrsboro, N.S. (07/27/91)
GHA[1]	*Melanoplus sanguinipes* (Orthoptera: Acrididae)[2]	unknown

[1] The GHA strain is the active ingredient in BotaniGard
[2] From Al Mazrá awi, 2007

The bioassay design, after Butt and Goettel (2000), included the aforementioned isolates of *B. bassiana*; and the GHA strain found in the commercial product BotaniGard (Koppert Canada), served as a positive control. BotaniGard and the GHA strain will be discussed as a treatment throughout, to distinguish it from the negative controls. ESB larvae were reared according to instructions given by Insect Production Services (The Canadian Forest Service, Sault Ste. Marie, ON), from which they were procured. Diapausing 2nd instars were cultivated in staggered groups on artificial diet (McMorran, 1965) procured from CEDARLANE, Burlington, ON (Appendix A2). Parafilm-gauze sheets containing the larvae were cut into pieces and each (containing 10-15 larvae) was

placed on the lid of an inverted 30 ml transparent plastic diet cup (Bioserv, acquired through CEDARLANE, Burlington, Ont.); the gauze faced the diet (Fig. 1). The larvae were placed in a Conviron® growth chamber (CMP 3244) maintained at 25^0C, ~75% relative humidity, and a 16h: 8h light: dark photoperiod. Head capsule measurements determined instar ages (McGugan, 1954), and upon reaching the targeted stadia ($4^{th}/5^{th}$ or 6^{th} instar), which took 9-14 days (Fig. 2), larvae were transferred to individual upright positioned diet cups prior to inoculation.

Figures 1 and 2. Budworm rearing. Second instar *Choristoneura fumiferana* larvae in a parafilm-gauze piece, on a 30 ml diet cup (inverted) lid (Fig. 1); 6^{th} instars grown in diet (Fig. 2).

To produce inocula, methods described by Goettel and Inglis (1997) were used. Conidia from the isolates 1756, B911, 2082, ARSEF and DAOM were scraped with a sterile metal inoculating loop from 18-day-old cultures into microcentrifuge tubes containing 400 µL of sterile distilled water and 100 µL of 10% Tween 80 solution (Sigma-Aldrich). One to 2 plates of each isolate were scraped depending on spore density. A sterile inoculating loop was used to break apart spore clumps and incidental mycelium in the first bioassay, and the tubes were subsequently vortexed for 15-20

seconds to suspend the spores. A handheld electric homogenizer was used for trials 2 and 3 instead of the inoculating loop, to break up spore clumps and mycelium. The instrument was sterilized and applied for 10-15 seconds on the inocula prior to vortexing. Five hundred additional microliters of sterile distilled water was added to the tubes after suspending the spores. A 10-fold dilution series for each isolate was then done. A series of microcentrifuge tubes (5) containing 900 μL of sterile distilled water were first prepared. The 10-fold dilution was done by pipetting 100 μL of the stock solution containing the spores into the first tube of the series; the same volume was then pipetted from that tube and into the next, until each tube had been inoculated. In this way, each tube contained 10 x fewer the spores than the solution in the tube preceding it. Between each 100 μL transfer, the tube from which the volume was taken was shaken to suspend the spores evenly. Conidia number was determined using an improved Neubauer haemocytometer. The standard inoculum concentration of 1.0×10^6 conidia would be applied to each larva in a 4 μL inoculum dose, so a concentration of 2.5×10^8 conidia/mL or 2.5×10^5 conidia/μL was required. The dilution tube comprising the closest concentration to this target was used for inoculum. Due to this methodology, the conidial preparations varied in concentration among the isolates (Table 2), but the same isolate suspension in a given bioassay was applied to both the 4^{th}/ 5^{th} and 6^{th} instar groups. Inocula concentrations (conidia/larva) are given in Table 2. The GHA strain (BotaniGard) was prepared as per instructions on the product bottle, and adjusted to a concentration of 2.5×10^5 conidia/μL.

Table 2. Inoculum concentrations for preparatory bioassays of *Beauveria bassiana* against *Choristoneura fumiferana.*

					Controls[3]
***B. bassiana* isolate**[1]	**Conidia /larva**	**Instar**	**Bioassay**	**n (#)**[2]	**n (#)**
ARSEF	2.00×10^6	4/5	1	16	18
ARSEF	1.10×10^6	4/5	2	19	17
ARSEF	1.10×10^6	4/5	3	20	22
B911	1.54×10^6	4/5	1	15	18
B911	1.20×10^6	4/5	2	15	18
B911	2.26×10^6	4/5	3	22	22
DAOM	1.10×10^5	4/5	1	19	15
DAOM	8.50×10^5	4/5	2	19	20
DAOM	3.00×10^6	4/5	3	18	22
1756	1.02×10^5	4/5	1	17	18
1756	1.07×10^6	4/5	2	17	13
1756	4.58×10^6	4/5	3	19	21
2082	7.70×10^5	4/5	1	19	19
2082	5.60×10^6	4/5	2	18	16
GHA (B)	1.00×10^6	4/5	2	19	19
ARSEF	2.00×10^6	6	1	24	23
ARSEF	1.10×10^6	6	2	22	22
ARSEF	1.10×10^6	6	3	22	22
B911	1.54×10^6	6	1	24	23
B911	1.20×10^6	6	2	22	22
B911	2.26×10^6	6	3	22	22
DAOM	1.10×10^5	6	1	24	22
DAOM	8.50×10^5	6	2	22	21
DAOM	3.00×10^6	6	3	22	22
1756	4.58×10^6	6	3	22	22
GHA	1.0×10^6	6	2	22	22

[1] See Table 1 for the source of these isolates
[2] Uneven sample sizes resulted from lack of larvae and test larvae being lost or damaged
[3] Controls were inoculated with 1% Tween 80 solution

Diet cups containing treatment and control larval groups were prepared. A mist of distilled water was sprayed on the diet cup lids immediately prior to larval inoculations to

produce adequate humidity for conidial germination. A micropipette was used to topically apply 4 µL of conidial suspension to the mid-dorsal surface (just behind the prothoracic shield) of treatment larvae. This direct method of inoculation is described by Butt and Goettel (2000), and Goettel and Inglis (1997). Control larvae were inoculated with 4 µL of 1% Tween 80 solution. The diet cups containing larvae were randomly positioned in cup trays and placed in a Conviron® growth chamber, set at 25^{0}C, ~75% relative humidity and a 16H: 8H light: dark photoperiod. Mean recorded conditions were 25.1 ± 0.1°C and 74.6 ± 2.7% RH (bioassay 1); 25.4 ± 0.4°C and 70.9 ± 4.8% RH (bioassay 2); and 25.5 ± 0.5°C and 56.6 ± 10.4% RH (bioassay 3). Control and treatment larvae were monitored for 10 days and assessed for mortality or pupation.

Purity and viability checks on inocula were also carried out as described by Goettel and Inglis (1997). When the larvae were treated, the same volume (4 µL) of conidial suspension was pipetted onto Sabouraud's dextrose and nutrient agar plates (Appendix A3)(Difco, VWR International, Mississauga, ON), which were incubated for 5 days in the same growth chamber as the larvae. The plates were examined daily for fungal growth and the presence of contaminating bacteria. To assess spore viability a 4 µL dose of conidial suspension was pipetted onto depression slides that were also placed in the growth chamber and percent germination was determined after 12, 24 and 36 hours.

Mortality checks of all larvae were carried out daily for 10 days post inoculation and cadavers were handled as described by Butt and Goettel (2000) and Goettel and Inglis, (1997). Aberrant behavior of larvae such as residing on the diet cup lid or in a feeding tunnel for extended periods, and other symptoms such as discoloration, and mummification or hardening of the body were noted. Cadaver halves were placed in

individual moisture chambers: 9 cm Petri dishes containing filter paper moistened with sterile water, and were monitored for the profuse sporulation characteristic of *B. bassiana.* The abdomen contents of remaining cadaver pieces were smeared in water on microscope slides and examined under phase contrast microscopy. Hyaline hyphae and hyphal bodies in the smears observed in conjunction with sporulation on the cadaver in the moisture chamber, confirmed mycosis. Spore mounts were also made, from cadavers exhibiting sporulation in order to identify the rachiform philaides and spore morphology characteristic of *B. bassiana.* The spore mounts were made at the end of the experiment at which time the abdomen contents of the remaining live larvae were also smeared in microscope slides, to determine if the pathogen was present internally.

Specific findings are given in the Results section but decisions made as a result of these preparatory experiments affected the next steps as discussed here. The isolates DAOM and ARSEF were selected for the mass culture work and the 6^{th} larval stadium of *C. fumiferana* was selected for use in subsequent bioassays. The electric homogenizer was excluded from the bioassay methodology; the Tween 80 solution concentration to be applied to control and treatment larvae (in inocula), was reduced to 0.05%, and methods were amended to ensure a standard inoculum concentration was applied to larvae in bioassays. A larval dose volume of 100 µL of inoculum with a target concentration of 1.0×10^7 conidia/ml (1.0×10^6 conidia per larva) was selected for subsequent bioassays.

Preparatory Investigation

Mass culturing *Beauveria bassiana* conidia on grain substrates:

Rationale

Two mass production experiments undertaken successively in May and June 2010 formed the basis of the methods used in the subsequent principal investigations. Methodology used was adapted from Bateman et al. (2004a), depending on the materials and space available. The essential components of this methodology have been used by Goettel (1984), Ibrahim and Low (1993) and Nelson et al. (1996) along with others. Main objectives were to explore the feasibility of using spent brewery grain as a mass culturing substrate, and compare two methods of broth inoculation (mycelial homogenate and spore suspension) to determine which most reliably produced large quantities of *B. bassiana* conidia (ARSEF and DAOM isolates) on grain. Other goals were to establish standard inoculation protocols, and determine incubation and drying arrangements for the grain cultures.

College of the North Atlantic, Carbonear, Newfoundland (product suppliers as at SMU)

Spent brewery grain as a mass-culturing substrate:

Eight collections of spent brewery grain from YellowBelly Brewery and Public House in St. John's, Newfoundland were retrieved (May and June, 2010) for potential use as a mass production substrate. The raw brewer's grain was baked for two days at 120^0C by the restaurant chef to reduce moisture and contaminants. Subsequently, the grain was

maintained at ambient laboratory temperature (25-30°C) in sealed plastic bags until it was tested (~10 days).

Rice and barley as mass culture substrates and inoculum production:

The first preparatory mass culture experiment investigated the feasibility of using a *B. bassiana* mycelial homogenate to inoculate Sabouraud's dextrose (SD) broth (Difco, VWR International, Mississauga, ON) (Appendix A4) and subsequently induce high sporulation on long grain white rice (Noname brand, Dominion, Carbonear, NL). DAOM and ARSEF isolates were cultured on SDA plates from cryopreserved spore suspensions prepared previously, and the cultures incubated for 3 days at ambient laboratory conditions (21.4 ± 0.3 °C and 32.5 ± 2.0% RH). The cultures including the agar were then homogenized with 25 ml of sterile water in a blender for 10-15 seconds. The homogenate was combined with 75 ml of sterile SD broth, contained in 250 ml sterile Erlenmeyer flasks. One *B. bassiana* culture was inoculated into one flask of broth, and this was the only provision toward standardized inoculum at this time.

The second preparatory investigation evaluated the potential of using a *B. bassiana* spore suspension to inoculate SD broth and subsequently produce high quantities of conidia on spent brewery grain and white rice. Seventeen-day-old sporulating cultures of the DAOM and ARSEF isolates established on SDA plates (from cryogenic vials), and incubated at ambient laboratory conditions (mean recordings were 21.4 ± 0.3°C and 32.1 ± 2.0% RH), were scraped and suspended in 50 mL of 0.05% Tween 80 solution. Inoculum concentration was determined using an improved Neubauer

haemocytometer, and adjusted to 6.0 x 10^6 spores/ml. One ml was pipetted into each Erlenmeyer flask containing 75 ml of prepared SD broth.

Controls for the experiments were prepared exactly as treatments but excluded the pathogen. The broth flasks for both experiments were sealed with sterilized rubber stoppers and to encourage rapid mycelium growth, they were placed on an orbital shaker at 150 revolutions per minute for three days, incubating at ambient laboratory temperature (25-30°C). The cultures were then used to inoculate prepared grain substrates; 75 ml of broth (1 flask) was dispensed into 500 g of grain.

To prepare the rice and spent brewery grain, the substrates were placed in a colander and rinsed under cold running water; the excess water was drained for 3-5 minutes. The grains were then heated in oil and water (300 ml of water and 20 ml of cooking oil per kg of grain) on a bench top burner. Peanut, maize or vegetable oil is suitable for this step (Bateman et al., 2004a). Noname brand vegetable oil (Dominion, Carbonear) was used in this experiment. Upon liquid absorption, 500-gram aliquots of the grains were weighed and transferred to autoclavable bags. Specialized autoclavable bags (21 x 8.25 x 4.75 inches) designed for growing fungal spawn (Mycosource Inc, 21 Maple Ave., Toronto, ONT.), which provided a microporous filter patch to facilitate air exchange and keep contaminants out, were used for the second preparatory mass culturing experiment (Sabbahi et al., 2007). The bags containing the 500 g aliquots of grain were folded over and after cooling to room temperature, were stored in totes, which were subsequently placed in a refrigerator maintained at 2-4°C over night. Each time totes were used throughout the experiments they were cleaned with dish detergent, rinsed, and sanitized with a 70% ethanol solution before new cultures were placed inside.

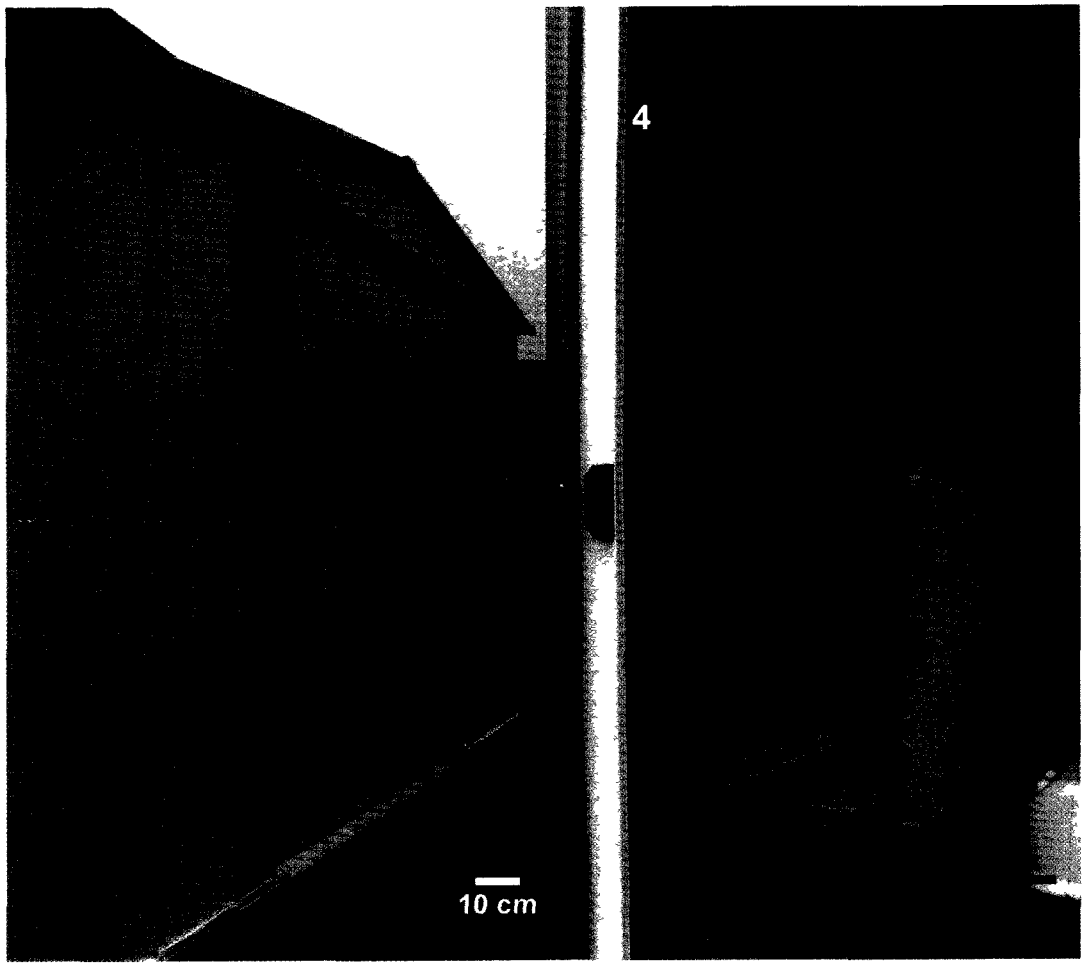
The following day the grain bags (via the totes) were transported to Memorial University of Newfoundland (MUN) (St. John's, NL) and were autoclaved at 121°C and 15 psi for 45 minutes. The bags were sealed upon removal from the autoclave. They were positioned in the totes again for transport back to the laboratory, and were placed in the refrigerator (2-4°C) over night, ready for inoculation the next day.

In a laminar flow hood the neck and mouth of each 250 ml flask was flamed and its contents poured into a grain bag. The bags were massaged from the outside to evenly distribute the inoculum and the openings were folded over loosely. The cultures were placed in sanitized plastic totes with ½ inch holes drilled in the sides to facilitate air exchange, and the tote lids (also cleaned and sanitized) were affixed. For experiment 1 (mycelial homogenate method), the containers were placed on shelves in a classroom closet lacking a ceiling, where they incubated for 14 days at ambient conditions to encourage mycelium colonization and sporulation on the grain (mean conditions were 23.0 ± 0.6°C and 27.1 ± 2.8% RH). In experiment 2 (spore suspension method), a Conviron® growth chamber (CMP5000) was used to incubate the cultures and was maintained at 25°C and 75% RH. Separate totes were used for treatments containing each *B. bassiana* isolate and for controls, but were placed adjacent to each other to assess cross contamination potential in these incubation settings. At the end of incubation the grain bags were opened and air-dried in a shed (sanitized with a 10% Clorox® solution) for 10 days at ambient conditions (means were 11.8 ± 3.6°C and 67.2 ± 10.0% RH (experiment 1); and 12.6 ± 4.2°C and 66.4 ± 10.0% RH (experiment 2). Totes were separated according to treatments and the lids were removed.

After inoculating the grain but prior to placing the cultures in incubation, grain samples were extracted aseptically to assess moisture content using a method described by Bateman et al. (2004a) involving a wet: dry weight comparison. The mass of each sample was initially found using small pre-weighed jars. They were subsequently baked in the jars at 80^0C for 24 hours and after cooling to room temperature, were re-weighed. Daily throughout the drying period, sporulation was assessed with microscopy. The presence/absence of spores was observed, and quantitative percentage estimates (percentage of grain particles covered with spores) were made.

Contamination checks were performed as well (Bateman et al., 2004a), using SDA plates that upon inoculation were incubated at ambient laboratory temperature (25-30°C) for 5 days. Checks included growing a sample of spore suspension and homogenized inoculum (~0.5 ml) on SDA before and after inoculating the broth. Microscopy checks for visible signs of contaminating fungi or bacteria were done on inoculated and un-inoculated broth, and 0.5 ml samples were also applied on SDA. Samples of inoculated and un-inoculated grain were also checked on SDA in the same way. Additionally, SDA plates were positioned randomly around the cultures during the incubation and drying stages to assess the presence of contaminants and the potential for cross contamination. SDA plates were placed outside the grain drying building as well, to determine the presence of *B. bassiana* in the natural environment. Specific instances where contamination occurred in the experiments will be explained where relevant. Grain cultures and controls were monitored daily for signs of contamination and/or sporulation of *B. bassiana* during incubation and drying. Culture dryness was assessed after 10 days using the same wet-dry weight comparison protocol described above.

Complications related to materials used in the preparatory mass culture experiments, occurred and it also became apparent that some methodology adjustments were needed. Particulars are given in the Results section, but several important choices are mentioned here. The building available for drying the grain cultures was inadequate because it could not be modified to promote drying conditions. A metal building that could be insulated with styrofoam and partitioned into sections was purchased, assembled and modified to become a drying house for the grain cultures. The building, 2.13 m x 3.05 m and obtained from Sears Canada, was assembled and positioned in a sheltered location on a nearby property (Figs. 3 and 4). Upon being erected, leveled and wired, it was lined (side, front and rear walls, ceiling and floor) with 1.40 cm thick insulating styrofoam cut to appropriate dimensions (Kent Building Supplies, Mount Pearl, NL). Tuck Tape® was used to attach the styrofoam sheets together and to surfaces, and Mono Ultra Exterior (a latex sealant) was applied to seal small remaining air spaces (Kent Building Supplies, Mount Pearl, NL). Styrofoam sheets were then used to partition the shed into three equivalent mini-rooms. One space was designated for controls and the remaining two were for treatments. The compartments were made as airtight as possible to minimize cross-contamination. Heavy plastic attached to the ceiling and upper portion of the room walls created an entrance to each space. The plastic was rolled back for entrance and the un-affixed plastic was sealed to the walls using clamps when access was not needed (Fig. 4). A space heater and dehumidifier were placed in the building to promote drying conditions. Hobo temperature/RH data loggers (Hoskin Scientific Ltd, Toronto) were used to monitor the conditions in the building, and prior to placing cultures inside, it was sanitized using a 10 % Clorox® solution.



Figures 3 and 4. Drying building for *B. bassiana* grain cultures (Fig. 3). Mini-rooms constructed with 1.40 cm thick insulating styrofoam (Fig. 4).

Other main decisions included the exclusion of spent brewery grain as a potential mass production substrate because of insufficient supply and the selection of pearled barley instead, in addition to long grain white rice. These substrates are referred to as 'rice' and 'barley' for the rest of this thesis. Rice and barley were supplied in bulk by G. J. Shortall Limited in St. John's, NL. The method used for the mycelial homogenate experiment for inoculating broth (experiment 1) was used in subsequent trials with two

modifications: incubation time of the starting cultures (3 days) was extended to 14 days to allow spore production, and standardized inocula (2.0×10^7 spores per broth flask) were administered. A second rotary shaker was obtained making it possible to double inoculations done at the same time. The autoclavable spawn bags were selected for subsequent trials, and were sealed with metal binder clips. The growth chamber was reserved for incubating the grain cultures. The principal investigations were similar to the preparatory studies except these changes were incorporated.

Principal Investigation

Mass culturing *Beauveria bassiana* conidia on long grain white rice and pearled barley:

Six mass-culturing experiments were undertaken in succession; the first three included the DAOM *B. bassiana* isolate and three utilized the ARSEF isolate. The fungus cultures were established on SDA (from cryopreserved spore suspensions) in 9 cm Petri dishes, and incubated for 14 days in the laboratory at ambient temperature (25-30°C). The cultures (including the agar) were then homogenized with 50 ml of 0.05 % Tween 80 solution in a blender, and conidia counts were determined using an improved Neubauer haemocytometer. The homogenate concentration was then adjusted to produce an inoculum with 1.0×10^7 conidia/ml, by combining it with 0.05 % Tween 80 solution. Two ml (a concentration of 2.0×10^7 conidia) were pipetted into 250 ml sterile Erlenmeyer flasks containing 75 ml of SD broth. The flasks were sealed with a sterile rubber stopper, and secured with sterile aluminum foil. Controls were managed exactly as treatments but excluded conidia. The flasks incubated on an orbital shaker at 150 revolutions per minute for three days at ambient laboratory temperature (25-30°C). One flask was used to inoculate 500 grams of prepared long grain white rice, and 500 grams of pearled barley, and each mass culture trial involved 12 bags of rice and 12 of barley.

Protocols described above were used to prepare the grains: rinsing, weighing into 500 g aliquots, heating, refrigerating and autoclaving for 45 minutes at 121°C and 15 psi (MUN). The broth inoculum was dispensed into autoclavable spawn bags containing the grain, which were then massaged from the outside for even inoculum distribution. The bag openings were folded over 2 to 3 times and sealed with sterile metal binder clips;

caution was taken in this step to leave the microporous filter patch un-obstructed. The grain cultures were then placed in a growth chamber maintained at 25°C and 75% RH for 14 days. Every second day the cultures were massaged to assist aeration, until incubation day 6.

Following 14 days of incubation, the grain cultures were removed from the growth chamber, placed in sanitized plastic totes and transported to the drying house. Treatments were placed in separate mini-rooms in the building according to grain type and isolate, while controls were placed in a single mini-room since building space was not sufficient for the construction of two control rooms. The bags were opened for drying and remained in this position for 7 days. Mean recorded building conditions were 19.3 ± 3.5°C and 59.2 ± 11.6% RH (trial 1); 28.0 ± 7.3°C and 43.4 ± 17.7% RH (trial 2); 19.3 ± 3.8°C and 53.7 ± 9.6% RH (trial 3); 22.0 ± 3.8°C and 41.1 ± 5.4% RH (trial 4); 22.3 ± 4.1°C and 46.4 ± 10.0% RH (trial 5); and 21.3 ± 3.8°C and 57.6 ± 15.8% RH (trial 6). When dried the bags of grain were sealed again with sterile metal binder clips; and transported back to the laboratory (via sterilized totes). Each grain bag was massaged to fragment culture clumps that would be problematic for harvesting. Mycelium and conidia extraction from the grains was accomplished with a MycoHarvestor-Version V (IC Consultants Limited, London, UK). This is a vacuum powered tool designed for separating powdery fungal spores from substrate; it functions to direct spores to one collecting tray and larger particles (substrate and mycelium) to a second tray. Substrates must be sufficiently dry for this machine to be used satisfactorily. The machine was cleaned and sterilized with a 70% ethanol solution, and each culture was placed in the fluid bed for 5 minutes. The products were subsequently placed into pre-weighed Ziploc

bags, and the yields determined. An attempt was made to run the control grains through the mycoharvester as well, however, these grains did not dry adequately and as a consequence clogged the machine. Contaminating yeast, which persisted in the controls, was thought to contribute to their retention of moisture. The controls had to be discarded in each trial.

Four grain cultures were discarded throughout the mass culture trials due to contamination during the drying phase: two from the DAOM isolate cultured on barley; and two from the ARSEF isolate cultured on rice. One bag of the ARSEF isolate grown on barley was lost because a broth flask migrated off the rotary shaker and broke on day 2 of incubation. Each trial would ideally produce 24 product bags (12 for mycelium and 12 for conidia) at harvest time, for a total of 144 (24 bags x 2 isolates x 3 replicates); but due to the discards the actual total was 134 bags. After being harvested, weighed, and bagged, the products were placed in a desiccator containing Drierite Anhydrous $CaSO_4$ desiccant for 7 days (bags open), and then into storage (Bateman et al., 2004a). For short-term storage, the bags were closed and placed in a sterilized desiccator that was subsequently placed in a fridge maintained at 2-4^{0}C.

Contamination checks on the homogenized inoculum before and after being placed in the broth and on inoculated and un-inoculated broth and grain were carried out as described previously. These checks included microscopic examinations as well as growing samples on SDA plates that were then incubated for 5 days at ambient laboratory temperatures (25-30^{o}C) (Bateman et al., 2004a). Prior to testing the pathogenicity of the mass cultured products against 6th instar Eastern Spruce Budworm larvae, due to low yields the harvested products for each grain in each mass culturing trial were combined.

A mass reduction occurred due to the final drying of the product in the desiccator, and areas of moisture developed in mycelium collections from bags 2, 4 and 5, from the 6th mass-culturing trial (a total of 12.2 g). This resulted in the discard of these harvests. The 131 remaining product bags were reduced to 12 with these integrations (1 bag x 2 grains x 2 isolates x 3 replicates). Due to very low production, yields harvested from the DAOM isolate and pearled barley, had to be combined. This further reduced the bags to 10, and each product formed a treatment in the pathogenicity bioassay (Table 3).

Analysis of Variance (ANOVA) (one-way, α=0.05) followed by simple linear regression analysis computed using R-Software (Chambers, 2008) (Version 2.12.2, Bell Laboratories: Luccent Technologies), was applied to compare yields among the isolate and grain combinations. R-Software was used for all statistical processing of the data. Like other parametric statistical tests, ANOVA assumes the data are drawn from a normal distribution (Goettel and Inglis, 1997). Samples must be random and independent and the homogeneity of variance must be satisfied. These assumptions were upheld by the data. ANOVA assessed the consistency of *B. bassiana* production among the trials, and identified weaknesses in the data prior to fitting a simple linear regression model. The regression indicated the most useful isolate and grain combination for producing the fungus. Two-way ANOVA was initially considered for this, but unequal replication within the dataset rendered this method inapplicable. The suitability of linear regression analysis lies within an existing relationship between the independent and dependent variables, normally distributed and independent errors, and constancy of error variance. In order to satisfy the assumption of constant error variance, a power transformation (square root) was applied to the data set. The assumptions were then satisfied as determined by

residual analyses. The number of conidia produced per gram of substrate was determined in conjunction with inoculum preparation procedures for the pathogenicity bioassays.

Table 3. Bioassay design for testing *Beauveria bassiana* against 6th instar *Choristoneura fumiferana.*

***Beauveria bassiana* isolate[1] (harvested conidia + mycelium)**	**Substrate**	**Trial**	**Mycelium and conidia bags combined[3]**	**Treatment in bioassay**	**n**
DAOM	Rice	1	12	A	25
DAOM	Rice	2	12	B	25
DAOM	Rice	3	12	C	25
DAOM	Barley	1, 2, 3[2]	29	D	25
ARSEF	Rice	4	10	E	25
ARSEF	Rice	5	8	F	25
ARSEF	Rice	6	12	G	25
ARSEF	Barley	4	12	H	25
ARSEF	Barley	5	12	I	25
ARSEF	Barley	6	12	J	25
DAOM (comparative control)	SDA	n/a	n/a	K	25
ARSEF(comparative control)	SDA	n/a	n/a	L	25
GHA-BotaniGard (positive control)	n/a	n/a	n/a	M	25
0.05% Tween 80 (negative control)	n/a	n/a	n/a	N	25
			T=131[4]		

[1] See Table 1 for sources of these isolates
[2] Low yields from the DAOM/barley treatment were combined to comprise treatment D
[3] Less than 36 bags in treatment D (12 x 3), and 12 in the other grain treatments (A-J), are due to discarded cultures
[4] A total of 131 product bags existed

Principal Investigation

Mass cultured *Beauveria bassiana* viability, and pathogenicity toward 6th instar *Choristoneura fumiferana* larvae:

Viability and pathogenicity of the mass cultured *B. bassiana* toward 6th instar *C. fumiferana* larvae were investigated following the mass production trials. Bioassay procedures as earlier described (Butt and Goettel, 2000; Goettel and Inglis, 1997) were also applied here. The assay compared the DAOM and ARSEF isolates cultured on grain and on agar, and the commercialized GHA *B. bassiana* strain, and was replicated three times (Table 3).

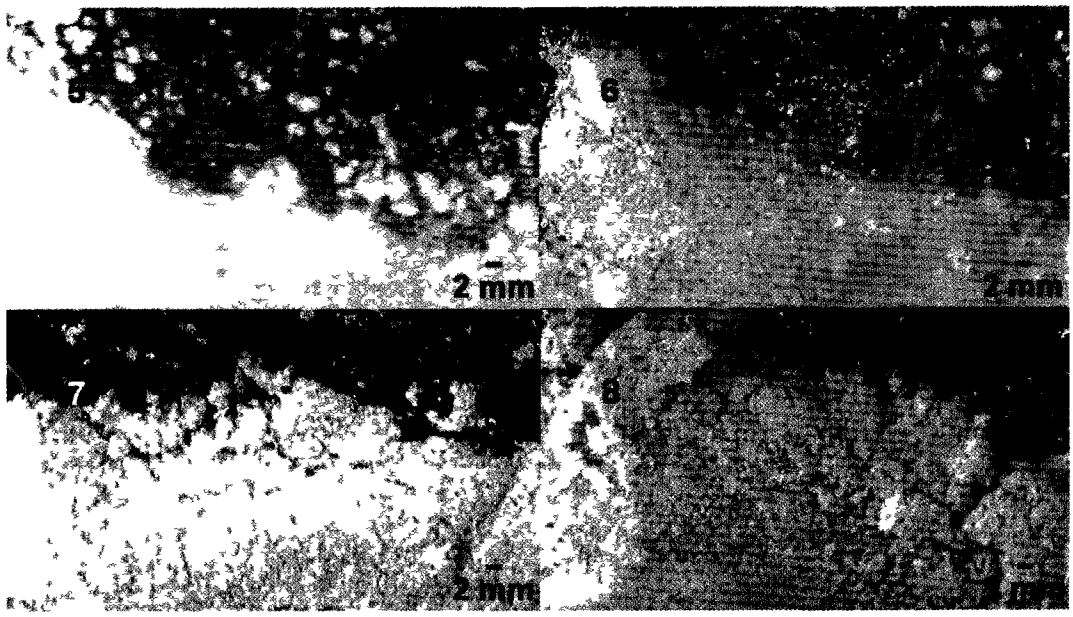
Larvae were reared to the 6th stadium on artificial diet. Twenty-five larvae were chosen randomly and treated using the protocol described previously. The standard inoculum concentration established for the bioassays was 6.0×10^6 conidia/ml based on the volume of product harvested when the DAOM *B. bassiana* isolate was cultured on pearled barley, the lowest yielding treatment. The entire yield was used to achieve this concentration, preventing replication of this treatment (Tables 3 and 4). The products were to varying degrees heterogeneous and coarse because of tiny grain particles harvested with the mycelium (especially with the DAOM treatments). In a laminar flow hood, a porcelain mortar and pestle set (135 ml) (WARD's Natural Science, St. Catherine's, ON) was used to mechanically crumble and homogenize the products (Table 4, Figs. 5-12). The mass produced ARSEF and DAOM isolates harvested from each trial were suspended in 0.05 % Tween 80 solution in 50 ml beakers (one per bioassay treatment), to produce the inocula (Table 4). Inocula derived from the agar grown isolates were prepared by suspending conidia from 14-day old cultures with 0.05 % Tween 80

solution also in 50 ml beakers. These cultures were grown from cryogenic preservations on SDA and were incubated at ambient laboratory conditions. Mean recordings were 24.4 ± 0.4°C and 44.9 ± 3.0% RH (bioassay 1); 25.0 ± 0.7°C and 43.2 ± 5.1% RH (bioassay 2); and 24.8 ± 0.8°C and 43.0 ± 7.0% RH (bioassay 3). Mycelial and conidial clumps were broken apart and further suspended using a sterile micro pestle. Conidial concentrations were then determined using an improved Neubauer haemocytometer and adjusted to 6.0 x 10^6 conidia/ml. BotaniGard was prepared according to manufacturer's instructions and adjusted to the target conidia concentration using sterile water. The negative control inoculum consisted of 0.05 % Tween 80 solution.

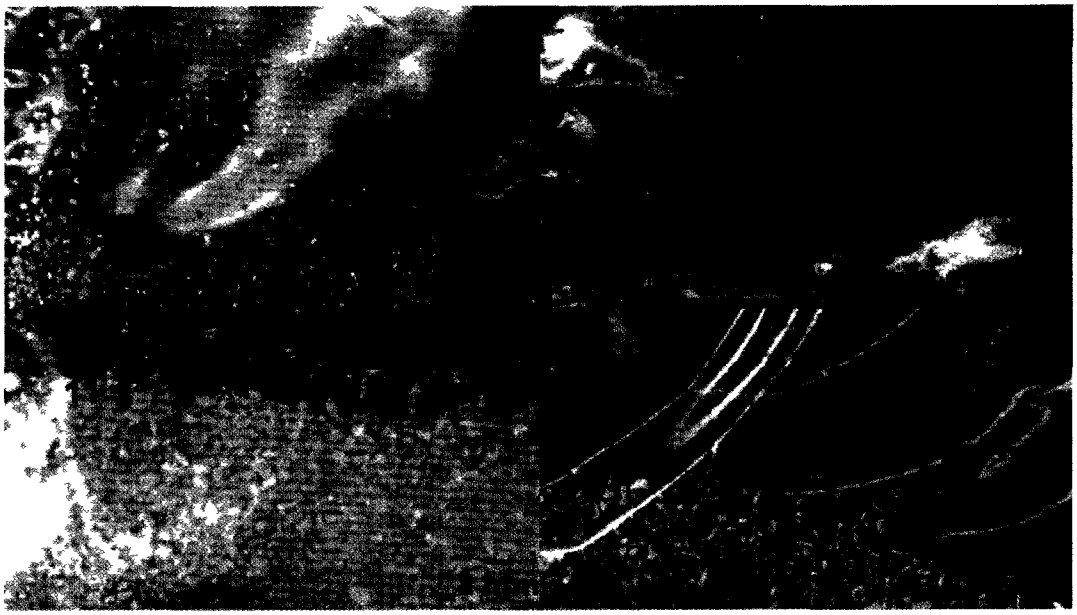
Table 4. *Beauveria bassiana* texture and conidia concentrations, when cultured on rice and barley.

				Conidia concentration potential		
Treatment in bioassay	**Trial**	***Beauveria bassiana* isolate[2] (conidia + mycelium) / grain**	**Product texture**	**Mass (g)**	**0.05% Tween 80 volume (ml)**	**~ Yielding inoculum concentration (conidia/ml)**
A	1	DAOM / rice	very coarse	1.50	50.00	6.0-6.5 x10^6
B	2	DAOM / rice	coarse	1.00	20.00	2.0-2.5 x 10^7
C	3	DAOM / rice	coarse	1.00	20.00	8.0-8.5 x 10^6
D	1, 2, 3[1]	DAOM / barley	very coarse	1.38	30.00	6.0 x 10^6
E	4	ARESF / rice	finest	0.10	20.00	3.0-3.5 x 10^7
F	5	ARESF / rice	finest	0.10	10.00	7.0-7.5 x 10^7
G	6	ARESF / rice	finest	0.10	20.00	4.0-4.5 x 10^7
H	4	ARSEF / barley	fine	0.10	40.00	6.0-6.5 x 10^7
I	5	ARSEF / barley	fine	0.30	20.00	1.0-1.5 x 10^7
J	6	ARSEF / barley	fine	0.10	40.00	4.0-4.5 x 10^7

[1] Low yields from the DAOM/barley treatment were combined to comprise treatment D
[2] See Table 1 for sources of these isolates



Figures 5-8. *Beauveria bassiana* (ARSEF, source in Table 1) culture on grains. Conidia from rice (Fig. 5), and barley (Fig. 6); mycelium from rice (Fig. 7), and barley (Fig. 8).



Figures 9-12. *Beauveria bassiana* (DAOM, source in Table 1) culture on grains. Conidia from rice (Fig. 9) and barley (Fig. 10); mycelium from rice (Fig. 11) and barley (Fig. 12).

Larvae were inoculated with a 100 µL dose each (a concentration of 6.0×10^5 conidia, except for controls which received 100 µL 0.05% Tween 80 solution) using the droplet application method previously described. Mortality checks were done as before for 10 days post inoculation; cadaver halves were placed in moisture chambers, and abdomen smears and spore mounts were made. Contamination and viability checks on the inocula using SDA, NA and depression slides were also done here. The Conviron growth chamber housing the treatment and control larvae was maintained at 25°C and 75% RH. Average recorded incubation conditions were 26.4 ± 0.9°C and 72.4 ± 4.5% RH (bioassay 1); 26.8 ± 1.1°C and 70.6 ± 5.7% RH (bioassay 2); and 26.8 ± 1.1°C and 69.7 ± 6.3% RH (bioassay 3).

ANOVA (one-way, $\alpha = 0.05$) was applied to compare percent mycosis mortality of ESB to assess the efficacy of the products (Goettel and Inglis, 1997). Time to death of each treatment was also examined by estimating LT_{50} values (time at which 50% of the treatment larvae had succumbed to mycosis). The data were not considered particularly strong for LT_{50} analysis, because in several of the treatment replicates in the bioassays, less than 50% of the larvae died from the fungus (Appendix C, Table C1). The DAOM/barley treatment as well was problematic, since it was only assayed once against ESB. While other analyses for time to death were first considered (LT_{50} using probit analysis and mean time to death using ANOVA), an estimate of LT_{50} using combined cumulative percent mycosis mortality was selected, to determine differences in virulence among the GHA *B. bassiana* strain, and the agar and grain grown DAOM and ARSEF isolates.

Results:

Opening note: *The results of the two preparatory investigations are presented first, and are followed by the results of the two principal investigations.*

Preparatory Investigation

Beauveria bassiana isolate sporulation and pathogenicity toward 4th, 5th and 6th instars of *Choristoneura fumiferana* larvae:

Microscopic and macroscopic observations indicated plentiful conidia on *B. bassiana* colonies established with ARSEF, DAOM and B911 isolates (from WSB, ESB, and blueberry leaftier respectively). Sporulation of the 1756 isolate (sawfly) was less prolific, and conidial production on the 2082 cultures (leaf beetle) was poor. Sporulation was asynchronous among these isolates but when incubated at 25°C, at 18 days conidia were present in acceptable quantities with some isolates, so this incubation period was selected as the standard culture method. The isolates also exhibited diverse gross colony morphologies. B911 produced abundant powdery spores and barely any mycelium, while 1756 and 2082 produced mostly mycelium and hardly any spores. Of the two isolates from spruce budworm, ARSEF seemed to produce more spores than mycelium, and DAOM cultures appeared to have spores and mycelium in equal quantities.

Growth tests on SDA and nutrient agar media performed on inocula used in the budworm pathogenicity bioassays, indicated they were viable and pure cultures; but the inocula in bioassays 2 and 3 did not produce as large colonies over the 5 day incubation period, in comparison to those of the first bioassay. Spore germination was also low for inocula in bioassays 2 and 3, and these tests performed for bioassay 1 failed due to procedural problems. There were six treatment exclusions across the 3 trials due to low

numbers of larvae from rearing (2082 four times and 1756 twice), while the commercial *B. bassiana* strain (GHA) was unavailable in trial 1 and was excluded from trial 3 due to a procedural error.

A total of 44 larvae (of 564) were displaced from the treatments in the three trials, and 41 of the displacements (93.2%) were $4^{th}/5^{th}$ instars. Such displacements also occurred in the control groups where 35 of the 43 escaped larvae were $4^{th}/5^{th}$ instars (81.4%), and the remaining 18.6% (8 of the 43) were 6^{th} instars. These displacements contributed to uneven sample sizes in the bioassays (Table 5).

For procedural reasons as described earlier the inoculum concentrations varied in these preparatory trials and this likely affected mortality levels. In some cases, such as with the ARSEF isolate in trial 1 which had high overall mortality, many more conidia (2.0×10^6 per larva) were applied than in other treatments in the same trial. The 2082 and B911 isolates in comparison to ARSEF, were applied at the lower rates of 7.70×10^5 and 1.54×10^6 conidia per larva respectively, and exhibited lower mortalities. The opposite however was also observed. The DAOM and 1756 isolates in trial 1 were applied at lower conidial doses (1.10×10^5 and 1.02×10^5 conidia per larva respectively) than other treatments in the same trial (2082 and B911, concentrations given above), and resulted in higher mortality than these treatments. Also, the results of the three trials were inconsistent. This is best exemplified by the 1756 isolate; in trial 3 when it was applied at 4.58×10^6 conidia per larva, the mortality was much lower (and 0 for the 4/5 instars) than in the first trial when it was applied at a rate of 1.02×10^5 conidia per larva. The overall mycosis mortality was markedly lower in trials 2 and 3 compared to trial 1 (Table 5), and of all the trials, the first was most consistent. So although the differences in conidia

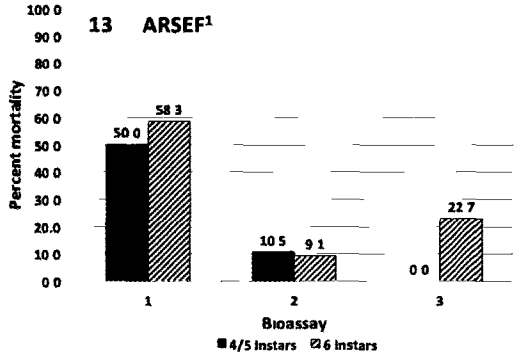
concentration may have had an influence on mycosis mortality, there must have been other factors that affected mortality. The discrepancy of mycosis death observed between trial 1, and trials 2 and 3 is thought to arise from the electric homogenizer used in the latter 2 trials to de-clump conidia and fragment mycelium. Sterilant used to clean the homogenizer, or over-homogenizing, may have damaged the spores reducing viability.

The problems encountered in the preparatory bioassays, and variable inocula concentrations used, impacted the results and precluded statistical analysis. However, pathogenicity caused by the *B. bassiana* isolates tested and the GHA strain was observed in 4^{th}, 5^{th} and 6^{th} instars in all the bioassays. The ARSEF isolate performed well in trial 1 causing 50.0 % mortality in the $4^{th}/5^{th}$ instars, and 58.3% in the 6^{th} instars. In trial 2, 10.5% and 9.1% of the $4^{th}/5^{th}$ and 6^{th} instar larvae respectively succumbed to mycosis, and in trial 3 while no mycosis deaths occurred in the $4/5^{th}$ instars, 22.7% of the 6^{th} instars succumbed to this isolate (Table 5, Fig. 13). The DAOM isolate behaved similarly, causing among the $4^{th}/5^{th}$ and 6^{th} instars respectively, 52.6% and 54.2% mortality (trial 1), 10.5% and 4.5% (trial 2) and 5.6% and 4.5% (trial 3) (Table 5, Fig. 14). Particularly evident in the DAOM isolate was pink pigmentation of cadavers (Figs. 19-20). This has been observed with *B. bassiana* (Zurek and Keddie, 2000) in cadavers of the satin moth, and may be an indication of oosporein production. An antiviral compound with red-pigmentation, oosporein has antibiotic activity as well (Zimmerman, 2007), and several *B. bassiana* strains are known to produce it (Eyal et al., 1994; Vining et al., 1962). The GHA strain caused the greatest mortality in the entire experiment, killing 84.2% of the $4^{th}/5^{th}$ instars and 86.4% of the 6^{th} instars (Table 5, Fig. 15).

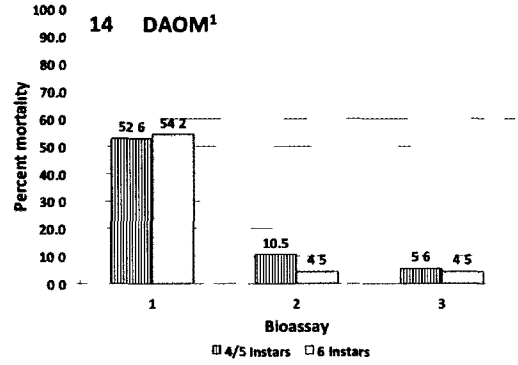
Table 5. Mortality of $4^{th}/5^{th}$ and 6^{th} instars of *Choristoneura fumiferana* from *Beauveria bassiana*.

							Controls		
***B. bassiana* Isolate[1]**	**Conidia /larva**	**Instar**	**Trial**	**n (#)**	**Other deaths (%)**	**Mycosis deaths (%)**	**n (#)**	**Other deaths (%)**	**Mycosis deaths (%)**
ARSEF	2.00×10^6	4/5	1	16	12.5	50.0	18	0.0	0.0
ARSEF	1.10×10^6	4/5	2	19	10.5	10.5	17	17.6	0.0
ARSEF	1.10×10^6	4/5	3	20	5.0	0.0	22	13.6	0.0
ARSEF	2.00×10^6	6	1	24	4.2	58.3	23	17.4	0.0
ARSEF	1.10×10^6	6	2	22	4.5	9.1	22	4.5	0.0
ARSEF	1.10×10^6	6	3	22	4.5	22.7	22	4.5	0.0
DAOM	1.10×10^5	4/5	1	19	5.3	52.6	15	20.0	0.0
DAOM	8.50×10^5	4/5	2	19	5.3	10.5	20	30.0	0.0
DAOM	3.00×10^6	4/5	3	18	0.0	5.6	22	27.3	0.0
DAOM	1.10×10^5	6	1	24	0.0	54.2	22	4.5	0.0
DAOM	8.50×10^5	6	2	22	4.5	4.5	21	0.0	0.0
DAOM	3.00×10^6	6	3	22	0.0	4.5	22	18.2	0.0
GHA	1.00×10^6	4/5	2	19	0.0	84.2	19	5.3	0.0
GHA	1.00×10^6	6	2	22	4.5	86.4	22	0.0	0.0
1756	1.02×10^5	4/5	1	17	11.8	64.7	18	5.6	0.0
1756	1.07×10^6	4/5	2	17	5.9	5.9	13	7.7	0.0
1756	4.58×10^6	4/5	3	19	15.8	0.0	21	4.8	0.0
1756	4.58×10^6	6	3	22	0.0	22.7	22	9.1	0.0
B911	1.54×10^6	4/5	1	15	26.7	33.3	18	22.2	0.0
B911	1.20×10^6	4/5	2	15	0.0	0.0	18	16.7	0.0
B911	2.26×10^6	4/5	3	22	4.5	9.1	22	9.1	0.0
B911	1.54×10^6	6	1	24	4.2	16.7	23	8.7	0.0
B911	1.20×10^6	6	2	22	4.5	9.1	22	0.0	0.0
B911	2.26×10^6	6	3	22	9.1	0.0	22	4.5	0.0
2082	7.70×10^5	4/5	1	19	15.8	15.8	19	15.8	0.0
2082	5.60×10^6	4/5	2	18	5.6	0.0	16	6.3	0.0

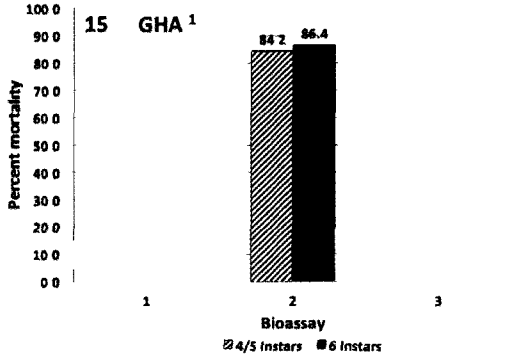
[1] See Table 1 for sources of these isolates



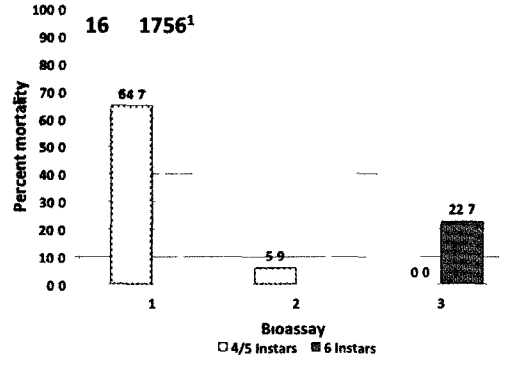
[1] See Table 1 for the source of this isolate inocula concentrations (conidia/larva) 2 00 x 10^6 (bioassay 1) 1 10 x 10^6 (bioassays 2 and 3)



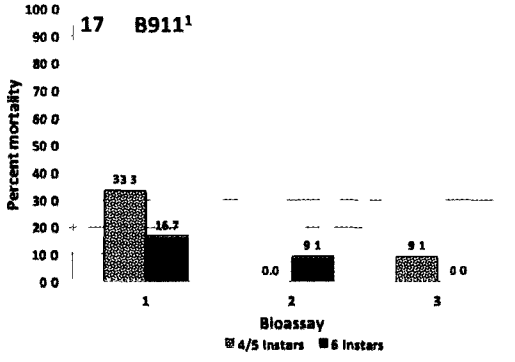
[1] See Table 1 for the source of this isolate inocula concentrations (conidia/larva) 1 10 x 10^5 (bioassay 1) 8 50 x 10^5 (bioassays 2) 3 00 x 10^6 (bioassay 3)



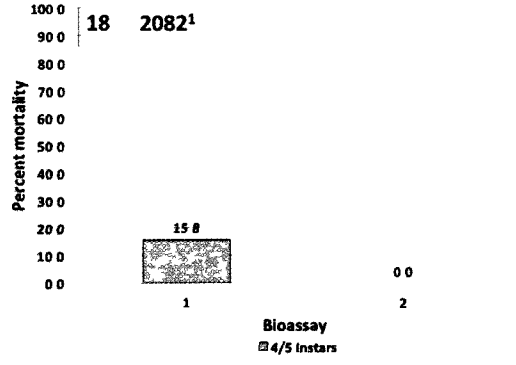
[1] See Table 1 for the source of this isolate the inoculum concentration (conidia/larva) was 1 00 x 10^6



[1] See Table 1 for the source of this isolate inocula concentrations (conidia/larva) 1 02 x 10^5 (bioassay 1) 1 07 x 10^6 (bioassays 2) and 4 58 x 10^6 (bioassay 3)

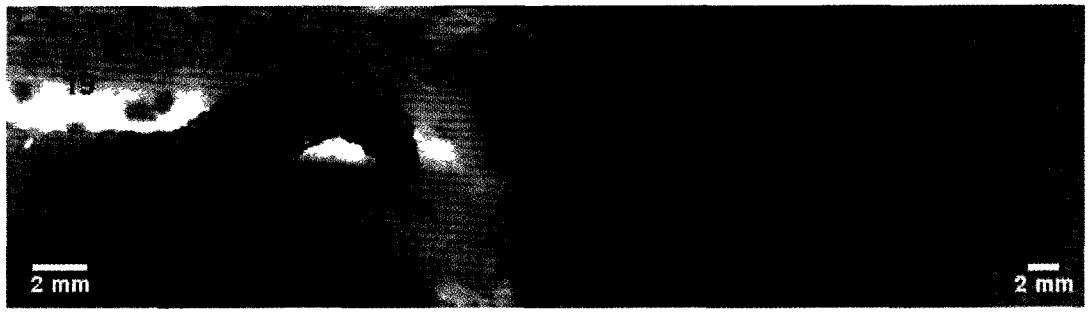


[1] See Table 1 for the source of this isolate inocula concentrations (conidia/larva) 1 54 x 10^6 (bioassay 1) 1 20 x 10^6 (bioassays 2) and 2 26 x 10^6 (bioassay 3)



[1] See Table 1 for the source of this isolate inocula concentrations (conidia/larva) 7 70 x 10^5 (bioassay 1) 5 60 x 10^6 (bioassays 2)

Figures 13-18. Percent mycosis mortality of *Choristoneura fumiferana* by *Beauveria bassiana.*



Figures 19 and 20. Symptoms of fungal infection by *Beauveria bassiana.* DAOM isolate (source in Table 1) infected cadaver (Fig. 19). Cadaver half on SDA showing pink pigmentation typical of DAOM (Fig. 20).

Of the non-spruce budworm isolates, 1756 seemed to out-perform the others; but a shortage in production of 6 instars precluded use of this isolate in trials 1 and 2 so observations were few. Among the $4^{th}/5^{th}$ instars in trials 1, 2 and 3 respectively, this isolate produced mortalities of 64.7%, 5.9% and 0%. When assayed against 6^{th} instars in trial 3, 22.7% of the larvae died from mycosis (Table 5, Fig. 16). The B911 isolate produced fairly low mortalities overall, 33.3 %, and 9.1% in the $4^{th}/5^{th}$ instars in trials 1 and 3 respectively; and when assayed against the 6^{th} instars, mortalities of 16.7% and 9.1% were produced in trials 1 and 2 respectively. No death caused by fungal disease from this isolate occurred in the $4^{th}/5^{th}$, and 6^{th} instar larvae in trials 2 and 3 respectively (Table 5, Fig. 17). The 2082 isolate was not tested on 6^{th} instars due to lack of larvae available at the time of the trials, and in trial 3 it was not tested on $4^{th}/5^{th}$ instars for the same reason. This isolate caused 15.8% and 0% mortality among the $4^{th}/5^{th}$ instar larvae in trials 1 and 2 respectively (Table 5, Fig.18).

Throughout the assays there was also some loss of larvae due to causes other than mycosis, mainly mechanical damage. This mortality was especially notable in the $4^{th}/5^{th}$

instars: 5-27% in all but three treatments. No such mortality occurred in the GHA and B911 treatments in trial 2, or in the DAOM treatment in trial 3 (Table 5). Among the 6^{th} instars no incidental mortalities occurred in the DAOM treatments in trials 1 and 3, or for 1756 in trial 3 (Table 5). In the remaining 6^{th} instar treatments there was a non-mycosis mortality of 4-5% except with B911 (trial 3), which had a mortality of 9.1% (Table 5). Overall a total of 8.1% (22/272) of the $4^{th}/5^{th}$ treated instars died for reasons other than mycosis, and 3.6% (9/248), of the treated 6^{th} instars. Non-mycosis mortality also occurred in the negative control larvae, especially the $4^{th}/5^{th}$ instars (Table 5). Overall, 38 of 278 $4^{th}/5^{th}$ instar control larvae (13.7 %) had non-mycosis deaths, compared to 16 of 243, 6^{th} instar control larvae (6.6%) that died incidentally (Table 5).

In considering how the bioassay outcomes would direct isolate and instar selections for the subsequent work, more emphasis was placed on the results of the first trial since the overall mortality was greater for this experiment. Also, although the inoculum checks conducted on SDA and NA were inconclusive, some defect in the inocula in trials 2 and 3 seemed probable. The greatest mycosis mortalities observed among the treatments in trial 1 were with the 1756 isolate (64.7% in the $4^{th}/5^{th}$ instars) at 1.02×10^5 conidia/larva; ARSEF (50.0% and 58.3 % in the $4^{th}/5^{th}$, and 6^{th} instars respectively) at 2.00×10^6 conidia/larva; and DAOM (52.6% and 54.2% in the $4^{th}/5^{th}$ and 6^{th} instars respectively) at 1.10×10^5 conidia/larva (Table 5). These isolates were selected for the subsequent work, with the highest priority being ARSEF and DAOM because they originated from *Choristoneura* spp., and exhibited better sporulation than the 1756 isolate. It was unclear as to which instar group, $4^{th}/5^{th}$ or 6^{th}, demonstrated a higher susceptibility to the pathogen, but unequivocally the $4^{th}/5^{th}$ instars were more

troublesome. These smaller instars escaped the diet cups often, molted which minimized the pathogen exposure to the host cuticle reducing opportunity for infection, and due to mechanical damage resulting from handling, non-mycosis mortality was higher among the smaller instars. For these reasons the 6^{th} stadium was selected for subsequent trials. The dose volume of 4 μL, which commonly slid off the larval cuticle upon inoculation, was increased to 100 μL for future bioassays to maximize exposure to the pathogen. The concentrations of inocula were adjusted so that each larva in each treatment received the same dose of conidia. The Tween 80 solution concentration applied to larvae in bioassays was adjusted to 0.05% to adhere to specifications of similar studies and because of the potential harm it could bring to larvae. The electric homogenizer was not used because it may have contributed to poor inoculum production.

Preparatory Investigation

Mass culturing *Beauveria bassiana* conidia on grain substrates:

Approximately 4 kg of spent brewery grain (in eight collections) was obtained from YellowBelly Brewery and Public House. The masses of grain acquired per trip ranged from 50 to 1500g, and the average mass per collection was 506.3 ± 494.8 g. This did not meet the substrate mass requirement per grain trial (6kg) that was established in conjunction with available equipment and materials. Macroscopic and microscopic examinations also revealed poor sporulation on the two 250 g spent brewery grain treatments included in the preparatory work (spore suspension method). The treatments had been inoculated with the DAOM and ARSEF *B. bassiana* isolates respectively. For these reasons, brewery grain was dropped as a mass culture substrate, and pearled barley was selected (in addition to rice). Pearled barley was chosen because its high surface area and structure would facilitate aeration and encourage sporulation (Bateman et al., 2004a; Masangkay et al., 2000). In addition, pearled barley could be obtained in sufficient quantities from a supplier.

Contamination, likely *Aspergillus* sp., which occurred during incubation, resulted in the discard of all the controls and 67% of the treatments in the first preparatory mass culture trial (mycelial homogenate method), and 33% of the treatments in the second trial (spore suspension inoculation method). All of the controls also had to be discarded in the second experiment as well; here contamination presented during the drying phase. The

shortened autoclave cycle (30 minutes as opposed to 45)[1] used in experiment 1, probably contributed to the contamination, as well as, the non-sterile environment in the incubation closet. The drying building used in the preparatory work also was note sterile and possibly augmented the contamination especially in experiment 2. One treatment (DAOM cultured on rice) in the first experiment remained sufficiently pure to be placed in the drying phase and four in the second experiment: two rice (DAOM and ARSEF) and two brewery grain treatments (DAOM and ARSEF). Average drying conditions in the building however were unfavorable: 11.8 ± 3.6°C and 67.2 ± 10.0% RH (experiment 1); and 12.6 ± 4.2°C and 66.4 ± 10.0% RH (experiment 2), and after 10 days the cultures were still wet and unsuitable for the MycoHarvester V fluid bed.

Due to the extent of contamination, little data were able to be collected from the preparatory trials. However, the methods and technical problems were addressed in the subsequent trials. Even with the technical difficulties, the treatment from the first experiment examining mycelial homogenate inoculum (DAOM cultured on rice), exhibited good sporulation and was the best of the treatments. Based on this result, this method was selected for the principal mass culture trials. Methods were amended to quantify the conidia with an improved Neubauer haemocytometer, thus enabling a standard concentration of spores to be inoculated into each broth flask in subsequent trials.

The closet used in experiment 1 for incubating the cultures did not perform well. It had an open ceiling and thus it was impossible to control for sterility, temperature, and

[1] Initial treatment grain in the first experiment was discarded due to an autoclaving technicality at Memorial University. Time and space resources limited grain preparation ability in the Carbonear laboratory after this incident.

relative humidity. Maintaining culture purity was especially a concern due to aged wooden shelves fixed in the closet, and the extensive use of the classroom within which it was located, on a daily basis. The growth chamber was selected for incubating the cultures in subsequent trials.

The drying building used for the preparatory mass culture work was also found unsuitable. This was mainly because the interior environment could not be controlled, and it was not possible to modify the structure to promote sterility and culture drying. With no physical barriers for separating the treatments, the building also did not support the experimental design. A new building was purchased (Sears Canada), assembled and modified as described in the Methods and Materials, to dry the cultures in subsequent trials. The results of the moisture analyses suggested that the liquid (broth) added to the rice cultures was sufficient.

Principal Investigation

Mass culturing *Beauveria bassiana* conidia on long grain white rice and pearled barley:

Total harvests from the grain cultures in each mass production trial (Appendix B, Tables B1 and B2) varied, the distributions showing no particular pattern except demonstrating ARSEF produced the highest yields when grown on rice (Table 6, Figs. 21-22). To determine if *B. bassiana* production was consistent throughout the trials, Analysis of Variance (one way, $\alpha = 0.05$) was performed. Total harvested product weights taken prior to the final drying phase were used for ANOVA instead of those subsequent to final drying. This is because as explained previously some mycelium collections had to discarded due to suspected contamination during the final drying phase. Total yields from the rice and barley produced by the DAOM isolate (trials 1, 2 and 3) were not statistically different (DAOM/rice: d.f. = 2, 15; F = 1.447; DAOM/barley: d.f. = 2, 13; F = 1.219). P-values of 0.266 and 0.327 respectively were found here (Table 6). When ARSEF was cultured on rice and barley in trials 4, 5 and 6 (ARSEF/rice: d.f. = 2,13; F = 5.574; ARSEF/barley: d.f. = 2, 14; F = 6.744), P-values of 0.018 and 0.009 respectively were found, indicating they were significantly different (Table 6). Bartlett's test ($\alpha = 0.05$) indicated variances for total product were equal in all treatments except for when ARSEF was cultured on barley ($k^2 = 6.338$; d.f. = 2, P = 0.041). Variances here were not statistically different at $\alpha = 0.01$ however. Conidia produced per gram of substrate for each treatment, calculated with information obtained during inocula preparations (Appendix D), are in Table 7.

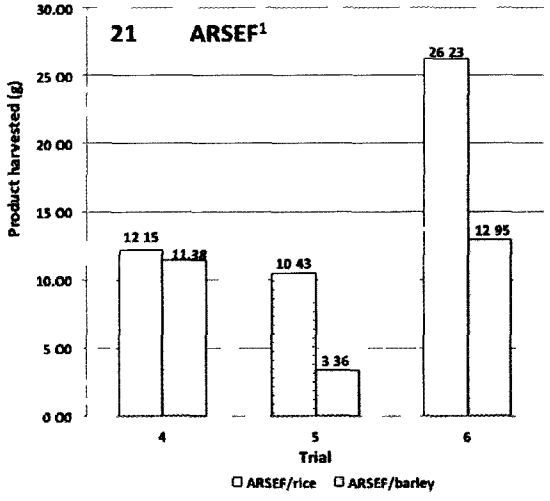
Table 6. Yields of *Beauveria bassiana* grown on grains.

			Mycelium			Conidia			Total = mycelium + conidia				
Treatment (*Beauveria bassiana* isolate[1] / grain)	**Trial**	**Total grain mass (kg)[2]**	**Yield (g)**	**Mean yield/500 g grain culture**	**Yield SD**	**Yield (g)**	**Mean yield/500 g grain culture**	**Yield SD**	**Yield (g)**	**Mean yield/ 500 g grain culture**	**Yield SD**	**P-value (total product)[3]**	**Total product dry mass (g)**
	1	3.0	6.48	1.08	0.46	0.12	0.02	0.02	6.60	1.10	0.46		5.34
DAOM /	2	3.0	5.38	0.90	0.51	0.11	0.02	0.01	5.49	0.92	0.52	0.266	3.47
rice	3	3.0	8.15	1.36	0.54	0.31	0.05	0.01	8.46	1.41	0.55		5.46
	1	3.0	0.62	0.10	0.02	0.00	0.00	0.00	0.62	0.10	0.02		0.39
DAOM /	2	2.0	0.50	0.13	0.06	0.02	0.01	0.01	0.52	0.13	0.06	0.327	0.40
barley	3	3.0	0.77	0.13	0.04	0.08	0.01	0.02	0.85	0.14	0.05		0.59
	4	2.5	11.37	2.27	1.12	0.78	0.16	0.14	12.15	2.43	1.17		8.86
ARSEF /	5	2.5	10.19	2.04	0.64	0.24	0.05	0.02	10.43	2.09	0.65	0.018*	7.25
rice	6	3.0	22.05	3.68	1.20	4.18	0.70	0.41	26.23	4.37	1.60		12.06
	4	3.0	8.14	1.36	0.22	3.24	0.54	0.25	11.38	1.90	0.40		8.45
ARSEF /	5	2.5	3.07	0.61	0.40	0.29	0.06	0.02	3.36	0.67	0.36	0.009*	2.09
barely	6	3.0	8.75	1.46	0.36	4.20	0.70	0.75	12.95	2.16	1.06		10.34

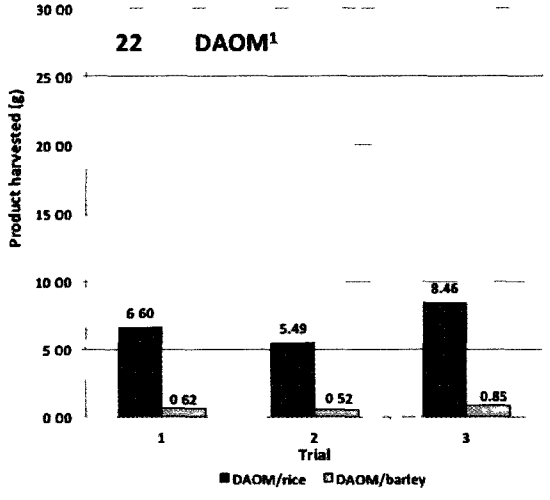
[1] See Table 1 for sources of these isolates

[2] Unequal grain masses are due to discarded cultures throughout the trials

[3] P-values obtained with ANOVA (α=0.05); an asterisk (*) following a P-value indicates significant difference in total harvests



[1] See Table 1 for the source of this isolate



[1] See Table 1 for the source of this isolate

Figures 21 and 22. Combined conidia and mycelium yields of *Beauveria bassiana* cultured on grains. Means and standard deviations are given in Table 6.

Table 7: *Beauveria bassiana* conidia produced per gram of grain.

			Conidia/g substrate	
Trial	***Beauveria bassiana* isolate[1] (conidia + mycelium) / grain**	**Conidia/g substrate**	**Mean**	**SD**
1	DAOM / rice	4.40×10^5		
2	DAOM / rice	7.30×10^5	5.40×10^5	1.65×10^5
3	DAOM / rice	4.50×10^5		
1,2,3	DAOM / barley	3.20×10^4	n/a[2]	n/a
4	ARESF / rice	2.90×10^7		
5	ARESF / rice	2.90×10^7	4.57×10^7	2.89×10^7
6	ARESF / rice	7.00×10^7		
4	ARSEF / barley	9.10×10^8		
5	ARSEF / barley	9.00×10^5	3.27×10^8	5.06×10^8
6	ARSEF / barley	6.90×10^7		

[1] See Table 1 for sources of these isolates
[2] Combining the DAOM product cultured on barley precluded obtaining a mean and standard deviation for this treatment

To determine which *B. bassiana* isolate and grain combination produced the most conidia, mycelium and total product (mycelium + conidia), a simple linear regression model was fitted to the entire data set. The associated coefficients and the R-squared values are given in Table 8. A linear relationship among mycelium, isolate and grain was indicated. The intercept of the fitted line was 0.973, and the R^2 value (0.779) indicated the regression was useful. When pearled barley was held constant, a 0.274 increase in mycelium per unit change in rice was expected. When ARSEF was held constant, mycelium production was expected to change by -0.282 per unit change in DAOM. These results indicate mycelium production was greatest when ARSEF was cultured on rice. When conidia production was examined in the model, only the isolate factor was

significant. The intercept of the fitted line was 0.535 and the R^2 value was 0.436. The expected change in conidia was -0.429 per unit change in DAOM when ARSEF was held constant. These results indicate ARSEF produced the most conidia regardless of the grain it was cultured on. In the model, a linear relationship among total production (conidia + mycelium), isolate and grain substrate was identified. The intercept of the fitted line was 1.109 and the R^2 value was 0.556. Holding pearled barley constant, a 0.458 change in total product per unit change in rice was expected; and when ARSEF was held constant, a change of -0.636 per unit change in DAOM was expected. From these results the rice and ARSEF combination gave the maximum total yield. Overall, ARSEF was the most prolific sporulating isolate producing the greatest amount of mycelium and total product on rice, and the greatest amount of conidia regardless of the grain substrate used (Tables 6 and 8; Figs. 21-22).

Table 8. Linear regression coefficients for *Beauveria bassiana* when cultured on grains.

		Coefficients				
Response		**Estimate**	**Std. error**	**t-value**	**Pr(>|t|)[3]**	**R-squared[4]**
Conidia	Intercept	0.535	0.051	10.521	$1.37 \times 10^{-15***}$	0.436
	Grain[1]	0.001	0.059	0.019	0.985	
	Isolate[2]	-0.429	0.059	-7.274	$6.15 \times 10^{-10***}$	
Mycelium	Intercept	0.973	0.022	45.080	$< 2.0 \times 10^{-16***}$	0.779
	Grain	0.274	0.025	10.920	$2.95 \times 10^{-16***}$	
	Isolate	-0.282	0.025	-11.240	$< 2.0 \times 10^{-16***}$	
Total product (conidia + mycelium)	Intercept	1.109	0.072	15.431	$< 2.0 \times 10^{-16***}$	0.556
	Grain	0.458	0.083	5.491	$7.35 \times 10^{-7***}$	
	Isolate	-0.636	0.083	-7.623	$1.49 \times 10^{-10***}$	

[1] Barley and rice
[2] See Table 1 for sources of these isolates
[3] Three asterisks (***) indicates highly significant
[4] As R-squared approaches 1, the utility of the regression increases

The texture and heterogeneity of the harvested yields were also examined (Table 4 and Figs. 5-12). When the DAOM *B. bassiana* isolate was cultured on rice and barley it was coarser and more heterogeneous in comparison to the ARSEF isolate. Furthermore when DAOM was cultured on barley, the product was coarser and more heterogeneous than when cultured on rice. Also, a greater amount of the DAOM harvested product was required to obtain a sufficient volume of inoculum in comparison to the ARSEF product. As an example 0.1 grams of the rice produced ARSEF isolate from the 6^{th} mass culture trial ('finest' consistency) and 20.0 ml of Tween 80 solution achieved a concentration of 4.0-4.5 x 10^7 conidia/ml. Ten times that mass (1.0 g) of the rice produced DAOM isolate from the 2^{nd} mass culturing trial ('coarse' consistency) with the same volume of Tween 80 solution, achieved a concentration of 2.0-2.5 x 10^7 conidia/ml. This was substantiated further in the quantification of conidia produced per gram of substrate; wherein higher numbers of conidia were obtained from the ARSEF isolate (Table 7). The ARSEF harvests were greater, contained more spores per gram of substrate, and were of a finer consistency.

Principal Investigation

Mass cultured *Beauveria bassiana* viability, and pathogenicity toward 6th instar *Choristoneura fumiferana* larvae:

The DAOM isolate mass cultured on barley was assayed only once against ESB. The data obtained from the three bioassays (Appendix C, Tables C1-C2) were approximately normal and had constant variance. Analysis of Variance (one-way, $\alpha = 0.05$) was applied to compare mycosis mortality caused by inocula prepared with the GHA *B. bassiana* strain, and with the isolates when cultured on agar, barley and rice. Significant differences were found (d.f = 6, 30; F = 5.428; P = 0.001). A Tukey's multiple comparison of means test ($\alpha = 0.05$), determined mortality caused by the DAOM isolate grown on rice to be significantly different from that of four other treatments: ARSEF isolate production on barley and SDA (P-values were 0.006 and 0.003); DAOM isolate production on SDA (P-value=0.016); and the GHA strain (P-value=0.030). In each case the DAOM isolate produced on rice caused less mortality (Tables 9-10).

Table 9: Mycosis mortalities of 6th instar *Choristoneura fumiferana* from *Beauveria bassiana*.

Treatment (*Beauveria bassiana* isolate[1]/grain)	**n**	**Mean mycosis death (%) (SE)**	**SD**
DAOM / rice	225	32.4 (6.05) a[2]	18.2
DAOM / barley	25	64.0[3] ab	
ARSEF / rice	222	54.8 (9.83) abc	29.5
ARSEF / barley	224	74.6 (8.48) bcd	25.4
DAOM / SDA	74	86.4 (5.00) bcde	8.7
ARSEF / SDA	73	95.8 (2.41) bcdef	4.2
GHA	75	82.7 (1.33)bcdef	2.3

[1] See Table 1 for the source of these isolates
[2] Values in columns with the same letter indicate non-significance at P< 0.05
[3] The barley produced DAOM isolate was tested in one bioassay

Table 10: Tukey's multiple comparison of means P-values for mycosis mortality.

Treatments compared (*Beauveria bassiana* isolate[1] / grain)		P-value[2]
DAOM / rice	DAOM / barley	0.828
DAOM / rice	ARSEF / rice	0.366
DAOM / rice	ARSEF / barley	0.006*
DAOM / rice	DAOM / SDA	0.016*
DAOM / rice	ARSEF / SDA	0.003*
DAOM / rice	GHA	0.030*
DAOM / barley	ARSEF / rice	1.000
DAOM / barley	ARSEF / barley	0.999
DAOM / barley	DAOM / SDA	0.975
DAOM / barley	ARSEF / SDA	0.875
DAOM / barley	GHA	0.990
ARSEF / rice	ARSEF / barley	0.509
ARSEF / rice	DAOM / SDA	0.366
ARSEF / rice	ARSEF / SDA	0.119
ARSEF / rice	GHA	0.513
ARSEF / barley	DAOM / SDA	0.984
ARSEF / barley	ARSEF / SDA	0.783
ARSEF / barley	GHA	0.998
DAOM / SDA	ARSEF / SDA	0.998
DAOM / SDA	GHA	1.000
ARSEF / SDA	GHA	0.990

[1] See Table 1 for the source of these isolates
[2] P-values with a * are significant at $P<0.05$

To obtain LT_{50} estimates (in days), cumulative percent mycosis mortality (over the 10 days of the experiment) for each treatment in each bioassay was determined, by combining all treatments pertaining to each grain and isolate in each bioassay (Microsoft Excel version 14.1.0). Since the data were approximately normal with constant variance, pooling it this way was valid. The day at which 50% of the larvae had succumbed to mycosis was the estimated treatment LT_{50} value. Up to 3 LT_{50} values therefore were obtained for each treatment (1 for each bioassay) and an estimate of mean LT_{50} (days)

among the values was then calculated (Table 11). In some treatments by the identified day more than 50% of the larvae had succumbed, and in several other cases less than 50% died from mycosis over the course of the experiment. This was especially the case for the DAOM isolate cultured on rice where less than half the larvae died from mycosis in the treatment replicates in each bioassay. In this analysis, the DAOM and ARSEF isolates cultured on agar, and the commercial *B. bassiana* strain (GHA) were shown to be the most virulent of all the inocula, killing the larvae in about 4-5 days. In comparison, for treatments based on the isolates produced on grain, mycosis deaths occurred 5 to 7 days after inoculation (Table 11).

Table 11. Estimated LT_{50} values for *Choristoneura fumiferana* when infected with *Beauveria bassiana*.

Treatment (*Beauveria bassiana* isolate[1] / grain)	**n**	**Mean LT_{50} (days) (SE)**
DAOM / rice	225	>10
DAOM / barley	25	6[2]
ARSEF / rice	148	6.5[3](1.5)
ARSEF / barley	224	5.3(0.88)
DAOM / SDA	74	4.3(0.33)
ARSEF / SDA	73	4.0(0)
GHA	75	4.7(0.3)

[1] See Table 1 for the source of these isolates
[2] The DAOM/barley LT_{50} value is based on one bioassay
[3] The ARSEF/rice value is based on two bioassays (1 and 2) since less than 50% of treatment larvae died from mycosis in bioassay

Discussion

Among the principal mass culture trials, production of the DAOM isolate was consistent regardless of which grain it was grown on, but Analysis of Variance showed ARSEF production on rice and barley were significantly different (P= 0.018; P= 0.009 respectively). When ARSEF was cultured on rice (3.0 kg) in trial 6 the total harvest was more than twice that collected from trials 4 and 5 (2.5 kg rice in each trial) (Table 6). When ARSEF was cultured on barley in trial 5 (2.5 kg) the yield was about a third of that from trials 4 and 6 (3.0 kg barley in each trial) (Table 6). The inconsistent production highlighted statistically for the ARSEF isolate, is attributed to these specific discrepancies. The unequal grain masses used for production in the respective trials (from discarded cultures) contributed, but do not seem sufficient to account entirely for the product variability that occurred; and there is no obvious explanation for it since culture method was consistent. Wyss et al. (2001) made the same observation in a study on mass production of an isolate of *Dactylaria higginsii* (Luttrell) (Orbiliaceae), a fungus used for weed control. High variability of sporulation on agar and grain media was speculated by Wyss et al. (2001) to be an aspect of somatic variability of the isolate, or variation in the growing conditions such as minor differences in essential nutrients, moisture and light. It is possible that these factors varied here as well, especially during the drying phases when relative humidity in particular, fluctuated. Production however of the DAOM isolate was consistent, with similar growing conditions.

Despite inconsistent production of the ARSEF isolate it was shown in the simple linear regression model to be more prolific than DAOM, producing the most conidia regardless of the grain substrate used, and the most mycelium and total product

(mycelium + conidia) on rice (Table 8). The ARSEF products were finer, more homogeneous and contained more conidia per gram of substrate (Tables 4 and 7; Figures 5-12). Overall, yields from barley were coarser than those from rice. When DAOM was cultured on barley the product was coarsest, and when ARSEF was cultured on rice they were finest. When ARSEF was cultured on barley, the product was still fine however. Product texture is associated with formulation viscosity. And since viscosity affects the compatibility of a mycoinsecticide with low and ultra low volume controlled droplet application equipment (motorized mist blowers and hydraulic sprayers for instance), texture is of paramount importance (Bateman et al., 2004b).

The isolates performed differently and had different morphologies. When grown on grain and agar ARSEF produced abundant powdery spores and less mycelium; and the DAOM isolate produced mainly mycelium and fewer spores. This variation in spore density and morphology is not uncommon. ARSEF could be among the isolates described by Feng et al. (1994), with suppressed vegetative growth and excessive sporulation, and the DAOM isolate could be of the opposite nature, or perhaps fits easily into neither classification. *Beauveria bassiana* conidiation has been studied by Wu et al. (2008), who report RNA binding proteins and 17 genes potentially associated with this process; but who maintain it to be a complex mechanism involving hundreds of genes, of which still little is known. Considerable genetic variability and heterogenicity occur naturally among *B. bassiana* isolates (Maurer et al., 1997; Padmavathi et al., 2003). Thus variation in spore density and morphology as observed between the ARSEF and DAOM isolates, is not surprising. These differences can be attributed to the genotypic variation among the isolates, and the possibility that some may have lacked a genotype encouraging

sporulation. How environmental stimuli (temperature, relative humidity, photoperiod) affect the mechanism of conidiation, and their function in triggering the switch between vegetative growth and sporulation is also yet to be determined (Zhang et al., 2009). It is possible that each isolate had a sporulation encouraging genotype, but that the correct environmental conditions to activate the involved genes were not provided. Nelson et al. (1996) for instance, affirm that optimal conditions can be different for each isolate.

This study and others on *B. bassiana* and other entomopathogenic fungi, found rice among various grains and other media, to be the best mass production substrate. Among the loose-solid media investigated (grated coconut flesh, mature papaya fruit, tapioca root, sweet potato tubers and rice grains) Ibrahim and Low (1993) discovered rice to be the best for growth and sporulation of *B. bassiana* and *Paceilomycess fumosoroseus*. Nelson et al. (1996) reported that of three grains (rice, barley and wheat) and two grain plus additives (rice + yeast, and rice + glucose) tested, rice alone supported the highest yields of *B. brongniartii* conidia.

The yields achieved in my study were not as high as previous studies with similar specifications. For instance, the greatest total yield (mycelium + conidia) harvested in the mass production trials came from ARSEF/rice cultures in the 6^{th} trial, where 26.23g (more than twice that harvested from the other treatments) were produced (Table 4). This equates to 0.009 grams of product per gram of rice, and this included mycelium (typically, conidia powder alone is discussed). Bateman et al. (2004a), suggest that about 0.04 grams of conidia powder per gram of substrate is typical of an efficient mass production system. Karanja et al. (2011) achieved a mean spore production of 7.38 g per 250 g of rice (0.03 grams conidia per gram of substrate), from an isolate of *B. bassiana*.

The production yields were low in terms of weight, but in terms of the number of conidia per gram of substrate (the maximum obtained here, 10^7 conidia per gram of rice for ARSEF) (Table 7), are lower but somewhat comparable to other studies. Feng et al. (1994) highlights *B. bassiana* production of 2.6×10^{11} conidia per gram of rice (Alves and Pereira, 1989), and *B. brongniartii* production of 1.0×10^8 (after 24 days) to 2.9×10^9 (after 42 days) on barley (Aregger, 1992). Goettel (1984) achieved *B. bassiana* conidial yields of 10^{10} per gram of wheat bran. And Nelson et al. (1996) produced 3.0×10^9 conidia of *B. brongniartii* per gram of rice, succeeding also with other substrates: ~1.3×10^9 condia per gram of barley; ~1.9×10^9 conidia per gram of wheat; 2.4×10^9 per gram of rice + yeast; and ~0.75×10^9 per gram of rice + glucose.

Cultivation time as an aspect of incubation period and, if applicable, drying duration, is an important consideration in mass production. The method used in this study (14 day incubation; 7 day drying), was similar to that described by Bateman et al. (2004a) and Jenkins et al. (1998) for producing *M. anisopliae*, and in consideration of preliminary trials, time restrictions and contamination potential. This method corresponds to the work of Goettel (1984) who used incubation and drying times of 14, and 4-5 days respectively for *B. bassiana*, and a method described by Feng et al. (1994) that included an incubation period of 12-15 days. Many researchers however, specify a longer incubation time. Ibrahim and Low (1993) and Karanja et al. (2011) with *B. bassiana*, and Nelson et al. (1996) with *B. brongniartii,* have reported high yields of the respective species, with 3-week incubation periods. Feng et al. (1994) indicated highest production of *B. brongniartii* conidia on shelled barley with a 4-week incubation period. And Masangkay et al. (2000) who studied *Alternaria alternata* (Fr.) (Pleosporales: Pleosporaceae) also

identified highest conidia production at 4 weeks. It is possible that if the isolates in this study had been subjected to a longer incubation period, this may have facilitated greater use of the available nutrients, and in turn encouraged higher spore densities. The MycoHarvester performed well for separating the spores and mycelium from the grains. However since it is not 100% efficient (Bateman et al. 2004a), some spores and mycelium could not be obtained, and this could have contributed to lower yields also.

Comparisons of mycosis mortality of the DAOM and ARSEF isolates produced on agar and grain, and the GHA *B. bassiana* strain, indicated that the DAOM isolate cultured on rice produced a lower mortality than the ARSEF isolate grown on barley or SDA, the DAOM isolate cultured on SDA, and the GHA strain (Tables 9 and 10). Mycosis mortality of the DAOM isolate cultured on rice from the first mass production trial was lower in the three bioassays, than this isolate cultured on rice in trials 2 and 3 (Appendix C, Table C1). In bioassay 2, several larvae (11 or 44%) treated with the DAOM isolate cultured on rice in trial 1, had non-mycosis deaths. Of this 44%, 9.1% (1 larva) exhibited only saprophytic *B. bassiana*, since the pathogen could not be identified within the abdomen; and 72.7 % (8 larvae) exhibited contaminating fungi on the cuticle (most likely *Aspergillus* sp.) along with saprophytic *B. bassiana*. Also in bioassay 2, several larvae (11 or 44%) treated with the DAOM isolate cultured on rice in trial 3 had non-mycosis deaths, and all of these larvae exhibited saprophytic *B. bassiana,* but again the pathogen could not be identified in the abdomen. Because this contamination occurred, and the absence of *B. bassiana* in the haemocoel prevented classifying these deaths as mycosis-induced, a lower overall mycosis mortality of this treatment was reported.

Butt and Goettel (2000) maintain that not all insects treated with a fungus die from infection, and while those that do not succumb are usually presumed to be healthy, this is not the case. In the non-mycosis deaths described above, *B. bassiana* was present on many of the larval cuticles and may have compromised the health of the larvae. It is also possible that *B. bassiana* induced mycosis, but the pathogen was subsequently outcompeted by the contaminating fungi and thus could not colonize the abdomen. The impurity which seemed inherent to this inoculum may have resulted from residual grain particles, and/or the product not having dried thoroughly, or it may have entered the system at some unknown juncture, such as the desiccator.

With the exception of the DAOM isolate cultured on rice, which produced a fairly low mortality (32.4 ± 6.05%), inocula from the other grain treatments, the agar grown isolates and the GHA strain, were reasonably high and statistically similar. The additional grain cultured isolates produced mortalities of: 64.0% (DAOM/barley); 54.8 ± 9.83% (ARSEF/rice); and 74.6 ± 8.48% (ARSEF/barley), but the mortality here for the DAOM/barley inocula was only based on one bioassay. These results indicate that pathogenicity of the isolates toward ESB was maintained throughout the mass production process. When grown on SDA, the isolates caused mortalities of 86.4 ± 5.00% (DAOM) and 95.8 ± 2.41% (ARSEF), and the GHA strain caused 82.7 ± 1.33% mortality. These were the highest levels of death in all the treatments, but were not statistically different from isolates grown on grain. This implies that inocula produced on barley and rice performed as well as conventional agar and the GHA strain, and therefore highlights these grains as viable substrates for producing quality spores of these isolates. The LT_{50}

estimations showed that the isolates cultured on agar and the GHA strain killed larvae faster than the grain grown isolates, suggesting they have higher virulence (Table 11).

Jackson (1997) maintains that a feasible large-scale production medium is typically based on the nutritional framework of a first established defined balanced medium, which is more expensive and not feasible for large-scale production (Masangkay et al., 2000). SDA, the defined, balanced medium used in this study to start *B. bassiana* cultures (Bateman et al., 2004a; Damir, 2006; Rangel et al., 2005; Safavi et al., 2007), and SDB used for the liquid phase, are nutrient rich (Ibrahim et al., 2002). Containing enzymatic digest of casein and animal tissue, which provide nitrogen and a complement of vitamins required for growth; and dextrose as an energy source, these media have all of the essentials to encourage fungal growth (Bateman et al., 2004a). As Rangel et al. (2006) suggest, dextrose or D-glucose is the most utilizable carbon source for fungi being readily incorporated into the cell. The mass culture media used, pearled barley and white rice are natural products, which have undefined nutrient status (Bateman et al., 2004a). Jenkins et al. (1998) substantiate that cereals such as these vary considerably in their nutrient status from crop to crop. Bateman et al. (2004a) emphasize the structure of the solid substrate in diphasic methods as being of critical importance, even more so than the nutrients it supplies; but Wyss et al. (2001), highlight the possibility for a medium to support heavy sporulation but not assure spore quality. Furthermore, it has been shown (Safavi et al., 2007; Wyss et al., 2001), that the type of growth medium used in mass production affects the number, stability, durability and virulence of fungal propagules. Several authors have pointed out that carbon sources, concentration, and carbon: nitrogen ratios, particularly affect spore yield and quality

(viability, virulence and stability) (Leland et al., 2005; Rangel et al., 2006; Shah et al., 2005; Shi et al., 2006; and Vega et al., 2003). Spore germinability for instance, has been observed to be influenced by media carbon concentration and carbon: nitrogen ratio (Wyss et al., 2001).

Rice and barley have undefined nutrient status but are rich in carbon. In this study, they may have contained residual nutrients from the agar (initially used to start the cultures), and the broth (the liquid phase), which were incorporated into them; although it cannot be determined whether nutrients were still available in the broth and agar at the time of incorporation. The primary carbon source in the grains however is a complex carbohydrate (starch), which is not particularly preferred or utilizable by the fungus. As ManHong and XingZhong (2006) emphasize, monosaccharides and disaccharides such as sucrose, trehalose, cellobiose, mannose and fructose, are preferred for growth and sporulation of *B. bassiana, M. anisopliae* and *Lecanicillium (Verticillium) lecanii* (Zimm.). Even so, adequate nutrition was provided to the fungus in the mass production system because spores were produced on the grains and were pathogenic toward ESB. The higher virulence indicated of the agar cultured isolates over the isolates produced on grain however, may be attributed to a faster germination rate on the host cuticle, and may be a factor of the production media.

Wu et al. (2010) observed that virulence of *M. anisopliae* conidia obtained from different substrates was significantly different when tested against DBM larvae. Safavi et al. (2007) attribute virulence differences of *B. bassiana* conidia on *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) larvae, to different production substrates. The connection between virulence and germination speed has been reported by Altre et al. (1999), who

observed isolates of *P. fumosoroseous,* which germinated fastest on the cuticle of DBM, to be most virulent. If the agar grown isolates here germinated faster on ESB larvae, this might explain why these isolates killed the larvae faster than the isolates produced on grain. The faster germination rate is probably connected to the nutritional completeness of the agar. Wyss et al. (2001) found the germination rate of *D. higginsii* conidia produced on rice to be substantially lower in comparison to conidia grown on Potato Dextrose Agar (PDA) and white rice amended with PDA. And similarly, Ibrahim et al. (2002) indicated nutrient rich media such as SDA and Yeast Extract Agar (YEA) encouraged greater germination of *M. anisopliae* conidia than minimal media. It has been suggested that the accumulation of lipid reserve, carbon or other sources of energy for early fungal development may be limited on rice (Wyss, 2001). Moreover, it seems that nutrient supplementation to the grains, may have produced spores with higher virulence.

An important consideration is that while higher rates of nutrients in a substrate can assist sporulation and conidial fitness, they can also have a limiting effect. Parisa et al. (2011) maintain that lack of or fewer nutrients can stress a growing fungus and affect germination speed, adhesion to the host cuticle and virulence of conidia. Exemplifying this, *M. anisopliae* conidia germinated faster and were more virulent on aphids (Ibrahim et al., 2002), and pollen beetles, *T. molitor* (Rangel et al., 2008) when minimal media was used as a production substrate. Wyss et al. (2001) observed that a low-glucose supplement to white rice increased conidiation, but a high-glucose supplement had an inhibitory effect.

Conclusions

The mass culture protocols used succeeded in producing very small yields of the DAOM (ESB) *B. bassiana* isolate, and small yields of the ARSEF (WSB) isolate on long grain white rice and pearled barley. The mass produced fungus was as efficacious toward ESB as the isolates grown on agar, and the GHA strain in BotaniGard, but was not as virulent. The DAOM isolate had very low and coarse yields, and the ARSEF isolate produced higher yields with finer consistency and more spores. ARSEF therefore is most promising for subsequent development. Further work on the DAOM isolate, particularly establishment of the requirements for triggering sporulation (for instance by manipulating nutrients and/or environmental conditions), may also render it more exploitable for mycoinsecticide incorporation. Both long grain white rice and pearled barley were determined to be viable substrates for mass production of the isolates studied, using diphasic methods. Rice produced higher overall quantities of *B. bassiana* and is therefore preferable to barley, but barley still performed fairly well particularly with the ARSEF isolate. The quantities of product cultured are suitable for laboratory and possibly greenhouse trials for the early stages of mycoinsecticide development. However the system is not particularly cheap, does not consistently produce high volumes of quality spores and is labor intensive. Although it does not represent a successful novel means for large-scale production, it can be improved and further developed.

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Appendix A

Media Composition

A1. Difco Sabouraud's Dextrose Agar (SDA)

Ingredients (Per Liter):
Enzymatic Digest of Casein (10.0g)
Dextrose (40.0g)
Agar (15.0g)
(Ingredients constitute a pale beige homogenous free-flowing powder)
Directions for preparation:
Dissolve 65.0 g of the powder in 1 liter of purified water. Heat agar media with frequent agitation (use magnetic stirir) and boil for 1 minute to completely dissolve the powder. Autoclave media at 121°C for 15 minutes.

A2. Modified McMorran Grisdale Diet (for spruce budworm)

Directions for preparing 1 liter:
Add 25.3 g of the provided agar to 835mL of cold water. Heat agar media with frequent agitation (use magnetic stirir) and boil for 1 minute to completely dissolve the powder. Pour agar solution into a blender and add 145.20 g of the dry mix. Add 5.1 g of the provided KOH solution. Blend the mixture well for 1 minute and dispense immediately.

A3. Difco Nutrient Agar

Ingredients (Per Liter)
Beef Extract (3.0g)
Peptone (5.0g)
Agar (15.0g)
(Ingredients constitute a tan homogenous free-flowing powder)
Directions for preparation:
Dissolve 23.0g g of the powder in 1 liter of purified water. Heat agar media with frequent agitation (use magnetic stirir) and boil for 1 minute to completely dissolve the powder. Autoclave media at 121°C for 15 minutes.

A4. Difco Sabouraud's Dextrose Broth

Ingredients (Per Liter)
Enzymatic Digest of Casein (10.0g)
Dextrose (20.0g)
(Ingredients constitute a pale beige homogenous free-flowing powder)
Directions for preparation:
Dissolve 65.0 g of the powder in 1 liter of purified water. Heat agar media with frequent agitation (use magnetic stirir) and boil for 1 minute to completely dissolve the powder. Autoclave media at 121°C for 15 minutes.

Appendix B

Beauveria bassiana production on grains

Table B1. *Beauveria bassiana* (DAOM isolate from ESB) production on grains.

			Harvested yields (g)		
MC[1] trial	**Grain bag (500 g/bag)[2]**	**Grain**	**Mycelium**	**Conidia**	**Total (conidia + mycelium)**
	1	rice	1.17	0.00	1.17
	2	rice	0.51	0.01	0.52
	3	rice	1.65	0.05	1.70
	4	rice	0.79	0.01	0.80
	5	rice	1.55	0.01	1.56
	6	rice	0.81	0.04	0.85
	7	barley	0.13	0.00	0.13
	8	barley	0.12	0.00	0.12
	9	barley	0.07	0.00	0.07
	10	barley	0.11	0.00	0.11
	11	barley	0.11	0.00	0.11
1	12	barley	0.08	0.00	0.08
	1	rice	0.38	0.01	0.39
	2	rice	0.59	0.01	0.60
	3	rice	0.84	0.02	0.86
	4	rice	0.81	0.01	0.82
	5	rice	0.91	0.02	0.93
	6	rice	1.85	0.04	1.89
	7	barley	0.08	0.01	0.09
	9	barley	0.09	0.01	0.10
	10	barley	0.11	0.00	0.11
2	11	barley	0.22	0.00	0.22
	1	rice	0.63	0.06	0.69
	2	rice	1.36	0.06	1.42
	3	rice	2.32	0.06	2.38
	4	rice	1.34	0.04	1.38
	5	rice	1.22	0.04	1.26
	6	rice	1.28	0.05	1.33
	7	barley	0.12	0.04	0.16
	8	barley	0.13	0.02	0.15
	9	barley	0.2	0.01	0.21
	10	barley	0.14	0.00	0.14
	11	barley	0.11	0.01	0.12
3	12	barley	0.07	0.00	0.07

[1]MC=mass culture

[2]Unequal replication is due to discarded cultures

Table B2. *Beauveria bassiana* (ARSEF isolate from WSB) production on grains.

			Harvested yields (g)		
MC[1] trial	**Grain bag (500 g/bag)[2]**	**Grain**	**Mycelium**	**Conidia**	**Total (conidia + mycelium)**
	1	rice	1.57	0.01	1.58
	3	rice	3.81	0.09	3.90
	4	rice	0.92	0.07	0.99
	5	rice	2.83	0.31	3.14
	6	rice	2.24	0.30	2.54
	7	barley	1.23	0.16	1.39
	8	barley	1.07	0.66	1.73
	9	barley	1.33	0.48	1.81
	10	barley	1.35	0.36	1.71
	11	barley	1.45	0.81	2.26
4	12	barley	1.71	0.77	2.48
	1	rice	1.93	0.01	1.94
	2	rice	2.67	0.06	2.73
	3	rice	1.16	0.06	1.22
	4	rice	1.76	0.05	1.81
	5	rice	2.67	0.06	2.73
	7	barley	0.52	0.06	0.58
	8	barley	1.18	0.08	1.26
	9	barley	0.47	0.07	0.54
	10	barley	0.25	0.05	0.30
5	12	barley	0.65	0.03	0.68
	1	rice	4.35	0.88	5.23
	2	rice	5.43	1.34	6.77
	3	rice	1.90	0.23	2.13
	4	rice	3.77	0.67	4.44
	5	rice	3.00	0.27	3.27
	6	rice	3.60	0.79	4.39
	7	barley	1.51	0.33	1.84
	8	barley	1.61	0.43	2.04
	9	barley	1.21	0.20	1.41
	10	barley	1.14	0.27	1.41
	11	barley	1.19	0.81	2.00
6	12	barley	2.09	2.16	4.25

[1]MC=mass culture

[2]Unequal replication is due to discarded cultures

Appendix C

Mortality data from bioassays

Table C1. Mycosis mortality of 6th instar *Choristoneura fumiferana* from mass produced *Beauveria bassiana*.

Bioassay	Treatment[1]	Mass-culturing trial	Grain substrate	n (#)[2]	Other deaths (%)	Mycosis deaths (%)
1	A	1	Rice	25	0.0	12.0
2	A	1	Rice	25	44.0	16.0
3	A	1	Rice	25	4.0	12.0
1	B	2	Rice	25	0.0	64.0
2	B	2	Rice	25	0.0	52.0
3	B	2	Rice	25	8.0	36.0
1	C	3	Rice	25	0.0	40.0
2	C	3	Rice	25	44.0	24.0
3	C	3	Rice	25	4.0	36.0
1	D	combined	barley	25	0.0	64.0
1	E	4	Rice	25	0.0	84.0
2	E	4	Rice	23	21.7	56.5
3	E	4	Rice	24	16.7	16.7
1	F	5	Rice	25	0.0	88.0
2	F	5	Rice	25	24.0	64.0
3	F	5	Rice	25	4.0	36.0
1	G	6	Rice	25	0.0	92.0
2	G	6	Rice	25	4.0	40.0
3	G	6	Rice	25	0.0	16.0
1	H	4	barley	25	0.0	100.0
2	H	4	barley	25	0.0	72.0
3	H	4	barley	25	0.0	64.0
1	I	5	barley	24	0.0	79.2
2	I	5	barley	25	12.0	68.0
3	I	5	barley	25	8.0	16.0
1	J	6	barley	25	0.0	100.0
2	J	6	barley	25	0.0	88.0
3	J	6	barley	25	0.0	84.0

[1] See Table 3 for the detail of these treatments

[2] Uneven sample sizes resulted from larvae escaping from the diet cups

Table C2. Mycosis mortality of 6th instar *Choristoneura fumiferana* from *Beauveria bassiana*.

Bioassay	Treatment[1]	Culture substrate	n (#)[2]	Other deaths (%)	Mycosis deaths (%)
1	K	SDA	25	0.0	96.0
2	K	SDA	24	4.2	79.2
3	K	SDA	25	0.0	84.0
1	L	SDA	24	0.0	95.8
2	L	SDA	24	0.0	91.7
3	L	SDA	25	0.0	100.0
1	M	n/a	25	0.0	84.0
2	M	n/a	25	0.0	80.0
3	M	n/a	25	0.0	84.0
1	N	n/a	25	8.0	0.0
2	N	n/a	25	28.0	0.0
3	N	n/a	25	20.0	0.0

[1] See Table 3 for the detail of these treatments
[2] Uneven sample sizes resulted from larvae being lost

Appendix D

Conidia per gram substrate calculation

Production information:

Mass culture trial.....	1
Mass culture substrate.....	rice
Total mass of substrate.....	3 kg (3000 g)
Total mass of conidia + mycelium harvested before final drying....	6.60g

Inoculum preparation information:

Product mass.....	1.5 g
Tween 80 volume.....	50.00 mL
Yielding conidial concentration.....	6.0 x 10^6 conidia/mL

Calculation:

50.0 mL x 6.0 x 10^6 conidia/mL = 3.0 x 10^8 conidia

3.0 x 10^8 conidia/1.5 g = 2.0 x 10^8 conidia/g product

2.0 x 10^8 conidia/g product x 6.60g = 1.32 x 10^9 conidia

1.32 x 10^9 conidia/ 3000g product = 4.4 x 10^5 conidia/g substrate